OXIDATION OF GASEOUS HYDROCARBONS BY ALKENE-UTILIZING BACTERIA

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Oxidation of gaseous hydrocarbons by alkene-utilizing bacteria

Proefschrift

ter verkrijging van de graad van
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1. De door Watkinson en Sommerville geschetste afbraakroute van 1,3-butadieen via pyruvaat is onjuist, ook in het licht van hun eigen experimenten.
   Watkinson and Sommerville, Proc Int Biodegradation Symposium (3rd) 1976 pp 35

2. De bewering dat met propeen als koolstof- en energiebron vooral mengculturen worden verkregen, is onjuist.
   Cerniglia et al, Appl. Environ. Microbiol. 1976 6 764

3. De veronderstelling dat de verhouding van enantiomeren van een door micro-organismen gevormd epoxyalkaan dezelfde is voor verschillende epoxyalkanen is onjuist.
   Furuhashi et al, Eur. J. Appl. Microbiol. 1981 12 39

4. Een goede operationele stabiliteit van geimmobiliseerde microörganismen wordt alleen verkregen indien de organismen omringd zijn door een compleet groeimedium
   Van Ginkel et al, Enzyme Microb. Technol. 1983 5 297

5. De opmerking van Burlingame and Chapman dat E. Coli stam C ook kan groeien op fenylazijnzuur als enige koolstof- en energiebron berust op een misverstand.
   Burlingame and Chapman, J. Bact. 1983 155 113
   Cooper and Skinner, J. Bact. 1980 143 302

6. Ook Gram-negatieve bacteriën zijn in staat op etheen als enige koolstof- en energiebron te gebruiken.
   Dit proefschrift

7. In hun drang de wereld te verbeteren maken idealisten gebruik van oogkleppen.

8. Een pessimistische visie werpt een realistischer licht op het wereldgebeuren dan een optimistische

9. Het in één ruimte plaatsen van een aantal personen leidt niet automatisch tot samenwerking.

Stellingen behorend bij het proefschrift "Oxidation of gaseous hydrocarbons by alkene-utilizing bacteria" van C.G. van Ginkel.

VOORWOORD

Het uiteindelijke resultaat van een promotieonderzoek "het proefschrift" is niet alleen het werk van de betrokken promovendus. Vandaar dat ik een aantal mensen wil noemen die bijdrage(n) hebben geleverd.

Allereerst Eric Welten, met wie ik anderhalf jaar nauw heb samengewerkt.
Jan de Bont voor de begeleiding tijdens het onderzoek.
De promotores W. Harder en A. Zehnder voor suggesties en het corrigeren van manuscripten.
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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

GENERAL

Hydrocarbons occur in two chemically distinct forms, namely as aliphatic and aromatic compounds. The former can be subdivided into alkanes (containing exclusively saturated carbon bonds), alkenes (with one or more double bonds) and alkynes (with at least one triple bond). Alkane and alkene molecules composed of up to four carbon atoms occur as gases at ambient temperatures and pressure. Aromatic compounds are under the same conditions either liquid or solid.

Largest sources of hydrocarbons are natural gas and petroleum. Gaseous alkanes are the main constituents of natural gas which is found in many areas of the world in porous reservoirs. The composition of natural gas varies widely depending on the area from which it is obtained. The methane content is usually between 50 and 90%, ethane between 5 and 20%, propane between 3 and 18% and butanes between 1 and 7%. Petroleum is a complex mixture of liquid and solid hydrocarbons; its composition also varies with the source. Both natural gas and petroleum are diagenic products from biomass.

Some hydrocarbons are biologically produced e.g. methane, ethene, isoprene and terpenes. Methane is the ultimate endproduct of anaerobic biodegradation of organic matter by a complex microbial population. This gas is commonly released from paddy fields, swamps, marches, gastrointestinal systems and sediments. Unsaturated hydrocarbons such as the plant hormone ethene is synthesized by many bacteria, fungi and plants (Abeles, 1973; Primrose, 1976), the volatile isoprene is emitted by leaves of some higher plants (Rasmussen, 1970), and large amounts of terpenoids are synthesized by many living organisms.

Gaseous hydrocarbons are also produced chemically. Ethene is manufactured in large amounts from feedstocks such as ethane or propane or from multicomponent feedstocks such as natural gas or naphtas which are made from petroleum. Today, ethene is produced almost exclusively via the pyrolysis of hydrocarbons. Other alkenes are produced primarily as by-products of petroleum refining and of ethene production. Ethene is the largest volume organic chemical produced today and it is converted by the petrochemical industry into a multitude of intermediate and end products, in particular polymeric materials such as plas-

tics.

Besides the emission of the biologically produced hydrocarbons, large amounts of gaseous bulk petrochemicals are released into the environment. As a consequence, these gaseous hydrocarbons occur abundantly.

Since there is much information about the biological and chemical behaviour of methane in the environment, its fate will be used here as an example. Most atmospheric methane is produced by anaerobic decomposition of organic matter as shown by measurements of the ^{14}C content in atmospheric methane (Ehhalt, 1975). However, most methane from biogenic sources is oxidized microbiologically before it reaches the atmosphere and this is reflected in the ubiquitous occurrence of methane-utilizing bacteria in the environment (Higgins et al, 1981). Methane can act as sole energy and carbon source for methane-oxidizers. However, these organisms can also oxidize some other hydrocarbons, but without being able to use them for proliferation. This so-called co-oxidation was originally reported by Leadbetter and Foster (1959) who showed that Pseudomonas methanica, an obligate methanotroph, would yield oxidation products from other gaseous alkanes if these alkanes were present during growth on methane. In recent years it has been shown that hydrocarbon-utilizing bacteria can introduce oxygen functions into a variety of hydrophobic organic compounds, many of which are of anthropogenic origin (Higgins et al, 1980) This oxygenation may allow, these otherwise recalcitrant compounds to be degraded by other micro-organisms. Hydrocarbon-utilizing bacteria fullfil therefore two environmentally important roles. Firstly, they maintain a low level of gaseous hydrocarbons in the atmosphere, and secondly, they "activate" certain compounds to make this biodegradation possible.

The scope of this thesis is to obtain a better insight into the microbial degradation of gaseous and volatile alkenes. Although the emphasis is on alkene-utilizing bacteria, some aspects of alkane-utilizing bacteria are included as well because these organisms can also transform certain alkenes. In the following our current knowledge on the microbial utilization and conversion of gaseous alkenes and alkanes is summerized and the consequent objectives for our research elucidated

OXIDATION OF GASEOUS HYDROCARBONS BY PROKARYOTES.

Methane-utilizing bacteria

Methane is a naturally occurring gas and it is therefore not surprising that bacteria have developed the ability to utilize methane as a carbon and energy source. Methane-utilizing bac-

teria are divided in two groups; (i) obligate methanotrophs with a type I membrane structure and the ribulose monophosphate cycle as the major carbon assimilation pathway (Methylococcus, Methylomonas and Methylobacter), (ii) obligate and facultative methanotrophs with a type II membrane structure assimilating carbon by way of the serine pathway (Methylosinus, Methylocystis and Methylobacterium) (Whittenbury et al, 1970; Higgins et al, 1981).

Methane mono-oxygenase is the enzyme responsible for the initial oxidation of methane to methanol. Evidence for the involvement of an oxygenase was reported by Higgins and Quayle (1970) who isolated $CH_3{}^{18}OH$ as a product of methane oxidation when suspensions of methane-grown bacteria were allowed to oxidize methane in an $^{18}O_2$-enriched atmosphere. The subsequent observations of methane-stimulated NADH oxidation catalysed by cell-free extracts of methane-utilizing bacteria (Ribbons and Michalover, 1970; Ferenci, 1974; Ribbons, 1975) suggested that the enzyme responsible for methane oxidation is a mono-oxygenase. Scott et al (1981) discovered that Methylosinus trichosporium OB3b possesses soluble and particulate methane mono-oxygenase activities, while Stanley et al (1983) and Burrows et al (1984) have shown that in methanotrophs the intracellular location of the methane mono-oxygenase is determined by the availability of copper. Particulate methane mono-oxygenase was found when the organisms were grown at relatively high copper biomass ratios, whereas organisms grown under conditions of copper deficiency contained mainly soluble methane mono-oxygenase. Methanol derived from methane, is subsequently oxidized to formaldehyde by a PQQ dependent methanol dehydrogenase (Duine and Frank Jzn, 1979) in all methane-utilizing bacteria tested, while Methylococcus capsulatus contains in addition a PQQ- plus NAD+-dependent dehydrogenase (Duine et al, 1984). PQQ-dependent methanol dehydrogenases oxidize primary alcohols, but often show either little or no activity towards secondary alcohols. These latter compounds are oxidized by a NAD+-dependent secondary alcohol dehydrogenase which has been detected in cell-free extracts of several methane-utilizing bacteria (Hou et al, 1979). Formaldehyde dehydrogenases found in methanotrophs can be classified into two groups; NAD(P)+-linked enzymes and NAD(P)+-independent enzymes that require an artificial electron acceptor (Attwood and Quayle, 1984). Finally, a NAD linked formate dehydrogenase has been found in several C_1-utilizing bacteria (Patel and Hoare, 1971).

Oxidation of gaseous hydrocarbons by methanotrophs

Cell-free extracts of methane-utilizing bacteria oxidize hydrocarbons in the presence of both NAD(P)H and oxygen.

Representatives of two groups of methanotrophs were examined. Colby et al (1977) investigated the mono-oxygenase of a type I methanotroph Methylococcus capsulatus (Bath). Gaseous alkenes were oxidized by the methane mono-oxygenase to their respective 1,2-epoxyalkanes, while internal alkenes such as cis- and trans-2-butene were oxidized to a mixture of 2,3-epoxybutane, crotonic alcohol and butanone. 1,3-Butadiene was oxidized to 1,2-epoxy-3-butene. Both primary and secondary alcohols were detected as products of the oxidation of the lower C2 to C4 alkanes by soluble methane mono-oxygenase of Methylococcus capsulatus (Bath). The C5 to C8 alkanes were also hydroxylated yielding a mixture of 1- and 2-alcohols but no 3- and 4-alcohols were formed. A representative of a type II methanotroph, Methylosinus trichosporium OB3b oxidized an identical range of substrates and this suggests that type I and type II methanotrophs possess similar mono-oxygenases (Stirling et al, 1978). Recent observations indicate that the substrate specificities of soluble and particulate methane mono-oxygenase differ in that the particulate methane mono-oxygenase is unable to oxidize aromatic and alicyclic compounds. Furthermore, soluble methane mono-oxygenase catalyses both terminal and subterminal hydroxylation of propane and butane whereas the particulate methane mono-oxygenase oxidizes propane and butane only to the secondary alcohols (Burrows et al, 1984).

Resting-cell suspensions of various methanotrophs grown on methane oxidize alkenes to the respective epoxyalkanes and alkanes to alcohols and these products accumulate in the supernatant. 1,2-Epoxybutane produced by Methylococcus capsulatus and Methylosinus trichosporium was racemic (Subramanian, 1986). Furthermore, methylketones, aldehydes and fatty acids were detected as products. Methylketones are formed from secondary alcohols while aldehydes and fatty acids are oxidation products of primary alcohols. The oxidation of these alcohols is mediated by enzymes already present in methane-grown bacteria i.e. methanol dehydrogenase, formaldehyde dehydrogenase and a secondary alcohol dehydrogenase. No appreciable differences in product formation were detected between various methanotrophs as shown in Table 1. Methylomonas methanica, however, appears to be more restricted in the range of substrates oxidized (Stirling et al, 1979).

Alkane-utilizing bacteria

Natural gas contains apart from methane also ethane, propane and butanes and several micro-organisms have been isolated that are able to grow on these gases. Organisms more or less taxonomically related as for instance Mycobacterium, Nocardia, Corynebacterium and Brevibacter are considered to constitute

the major alkane-utilizers (Fuhs 1961, Foster 1963, Patel et al, 1983a).

The initial oxidative attack on alkanes involves molecular oxygen with the formation of primary alcohols as first products. These alcohols are converted by primary alcohol dehydrogenases to their corresponding aldehydes and subsequently aldehydes are oxidized to fatty acids (Fig. 1). This major

Table 1. Rate of formation of products from gaseous hydrocarbons by resting-cell suspensions of a type I and type II methanotroph grown on methane, Arthrobacter CRL60 grown on propane or butane and Mycobacterium E3 grown on ethene.

Organism		Methylococcus caps. 1)	Methylosinus trichosporium 1)	Arthrobacter CRL60 2,3)	Mycobacterium E3 4)
Growth substrate		Methane	Methane	Butane/ Propane	Ethene
Substrate	Products	Formation rate nmol min^{-1} (mg protein)$^{-1}$			
Ethene	1,2-epoxyethane	33	16	32	0
Propene	1,2-epoxypropane	33	30	42	16
1-Butene	1,2-epoxybutane	11	4	2	14
2-Butene	2-Buten-1-ol	3	3	-	0
	2,3-epoxybutane	6	6	-	20
Butadiene	1,2-epoxybutene	17	23	1	-
Ethane	ethanol	33	22	-	0
Propane	1-propanol	2	2	0	0
	propanal	3	2	0	0
	2-propanol	17	10	20	0
	acetone	42	25	23	0

- not determined

1) Hou et al, 1980 2) Patel et al, 1983a 3) Patel et al, 1983b 4) Habets-Crützen et al, 1984

oxidized to fatty acids (Fig. 1). This major route of alkane oxidation is not used in most propane-utilizing bacteria since the principal route of propane dissimilation is via 2-propanol which is further converted to acetone as shown in Fig. 1 (Lukins and Foster, 1963). This metabolic sequence was confirmed by investigating the presence of isocitrate lyase activity in variously-grown cells. The enzyme was induced in cells grown on propane and 2-propanol whereas 1-propanol-grown cells

did not contain isocitrate lyase activity (Vestal and Perry, 1969). Nevertheless, Stephens and Dalton (1986) claimed that the terminal oxidation pathway in their organisms is more important than previously supposed on the basis of simultaneous adaptation experiments. Acetone which originates from isopropanol oxidation, is probably metabolized via acetol to either pyruvate (Taylor et al, 1980) or via an unknown intermediate to acetate and a C_1 fragment (Goepfert, 1941; Levine and Krampfitz, 1952; Vestal and Perry, 1969; Hartmans and de Bont, 1986).

Oxidation of gaseous hydrocarbons by alkane-utilizers

As in methanotrophs, the oxidation of hydrocarbons by cell-free extracts of alkane-grown bacteria is dependent upon the presence of oxygen and reduced NAD. Both primary and secondary alcohols have been detected as products of propane and butane oxidation (Patel et al, 1983a). Cell-free extracts of propane-grown Brevibacterium sp. oxidized gaseous alkenes to their respective 1,2-epoxyalkanes (Hou et al, 1983; Patel et al, 1983b), whereas the formation of unsaturated alcohols from alkenes was not detected.

In general, resting cells of alkane-grown bacteria are able to hydroxylate alkanes and to epoxidize as well as hydroxylate alkenes. Oxidation of alkanes by washed cell suspensions of alkane-grown bacteria yields both primary and secondary alcohols as well as aldehydes and ketones (Patel et al 1983a). The epoxidation of volatile 1-alkenes by alkane-grown (C_6-C_8) bacteria was first demonstrated by van der Linden (1963), and later on confirmed by Cardini and Jurtchuk (1970), Abbott and Hou (1973) and May et al (1975). However, the mono-oxygenase of Pseudomonas oleovorans when given gaseous alkenes was only able to hydroxylate and did not epoxidate these compounds (May et al, 1975). Cell suspensions of organisms grown on lower alkanes (C_2-C_4), however, were able to catalyse the epoxidation of ethene, propene, 1-butene, 1,3-butadiene and 1-pentene. Epoxyalkane formation by such bacteria has been observed frequently (Hou et al, 1983; Patel et al, 1983b) and it was shown that the stoichiometry of consumption of propene and the production of 1,2-epoxypropane was approximately 1.2 : 1 (Patel et al, 1983b). The series of oxidation products formed from gaseous hydrocarbons by the bacteria grown on lower alkanes were very similar to the products formed by washed cell suspensions of methane-grown bacteria and product formation rates were also comparable (Table 1).

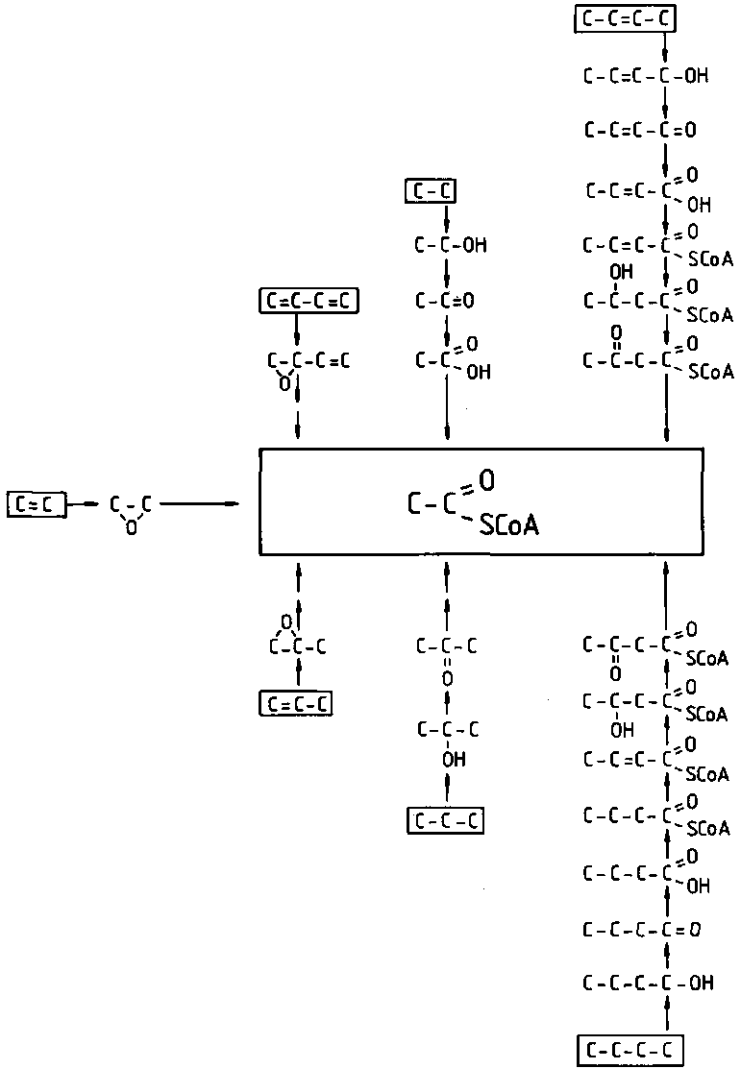

Figure 1. Scheme for degradative routes of several gaseous hydrocarbons as observed in various bacteria.

Alkene-utilizing bacteria

The plant hormone ethene (Abeles, 1973) and the volatile compound isoprene (Rasmussen, 1970) are the only naturally ocurring lower alkenes and these, as well as many other alke-

nes, are manufactured chemically in large quantities. Bacteria able to grow on these compounds have been isolated and they belong to the genera Mycobacterium and Nocardia (Heyer, 1976; de Bont, 1976; Watkinson and Sommerville, 1976; Cerniglia et al, 1976; de Bont et al, 1980).

Mono-oxygenases are responsible for the initial oxidation of alkenes (Watkinson and Sommerville, 1976; de Bont and Harder, 1978) to the corresponding 1,2-epoxyalkanes (Fig. 1). Information about the further metabolism of alkenes is scarce. 1,2-Epoxyethane is converted directly into acetyl-CoA by a dehydrogenase (de Bont et al, 1979) and many other alkenes are also metabolized via acetate as shown by increased isocitrate lyase activities and fatty acid profiles (Watkinson and Sommerville, 1976; Cerniglia et al, 1976).

Oxidation of gaseous hydrocarbons by alkene-utilizers

Cell-free extracts of alkene-grown bacteria oxidized alkenes when both molecular oxygen and NADH were present (de Bont and Harder, 1978). Several alkenes were oxidized by washed cell suspensions of alkene-grown bacteria and due to a broad substrate specificity of the mono-oxygenase also alkenes that do not serve as growth substrates were oxidized (de Bont et al, 1979). Formation of epoxyalkanes from certain alkenes by these bacteria occurred because the epoxide degrading enzymes had a more restricted substrate specificity (Habets-Crützen et al, 1984). Some of these alkene-grown bacteria produce chiral epoxyalkanes (Habets-Crützen et al, 1985). However, alkanes were not attacked by washed cell suspensions of alkene-grown bacteria (Fig. 2) and in this respect alkene-utilizing bacteria are exceptional (de Bont et al, 1979).

Alcohol- and ketone-grown bacteria

In general, hydrocarbon oxidation by micro-organisms is induced in the presence of hydrocarbons, but induction by non-hydrocarbon substrates has also been reported. For instance Mycobacterium paraffinicum and Mycobacterium JOB 5 did not lose the ability to oxidize gaseous alkanes when grown on ethanol or acetone, respectively (Davis et al, 1956; Lukins and Foster, 1963). Recently, Stephens and Dalton (1986) showed that 1-propanol-grown Arthrobacter B3aP oxidized propane. Published reports on alkane and alkene oxidation by alcohol- and acetone-grown bacteria are scarce or absent. The oxidation of ethane and propane by such cells was demonstrated by the enhancement of oxygen uptake rates following their addition to washed cell suspensions (Davis et al, 1956; Lukins and Foster, 1963; Stephens and Dalton, 1986).

Nitrifying bacteria

Oxidation of ammonia to nitrate in nature is a microbial process and bacteria responsible are for instance the ammonia oxidizer Nitrosomonas and the nitrite oxidizer Nitrobacter. The initial oxidation of ammonia by the chemolithotroph Nitrosomonas spp. involves the incorporation of molecular oxygen and the most appropriate name for the enzyme catalysing this reaction is ammonia mono-oxygenase. During normal growth the product of ammonia mono-oxygenase, hydroxylamine, is oxidized further in several steps to the endproduct nitrite.

Nitrosomonas spp. is capable of oxidizing methane to methanol and evidence was presented that ammonia mono-oxygenase catalysed this reaction (Hyman and Wood, 1983). Likewise, the ammonia mono-oxygenase oxidized gaseous alkenes like ethene and propene. Incubation of Nitrosomonas europaea cells with either ethene or propene led to the formation of the respective 1,2-epoxyalkanes (Drodz, 1980, Hyman and Wood, 1984).

OXIDATION OF GASEOUS HYDROCARBONS BY EUKARYOTES

Yeast and fungi

Several yeast are capable of growing very well on decane and higher n-alkanes but growth on gaseous alkanes by yeasts has not been observed. There is in fact one exception; Wolf and Hanson (1979, 1980) reported the isolation of a few yeast strains that could utilize methane, ethane and butane as carbon and energy sources. However these yeasts were subsequently lost. Utilization of gaseous hydrocarbons by filamentous fungi is probably more wide-spread. Zajic et al (1969) isolated a fungus which was tentatively identified as a Graphium sp. that grew on natural gas. Ethane appeared to be the sole carbon and energy source for the fungus, whereas methane was cometabolized (Volesky and Zajic, 1971). Fungi of other genera as for instance Acremonium, Penicillium and Allescheria are also able to utilize gaseous n-alkanes (McLee et al, 1972; Davies et al, 1973) The microsomal fraction of cell-free extracts of ethane-grown Acremonium sp. oxidized ethane only in the presence of NADPH as cofactor suggesting that a mono-oxygenase is involved (Davies et al, 1976).

Plants

Ethene is a natural plant growth regulator involved in the control of a wide range of developmental responses such as growth abscission and fruit ripening (Abeles, 1973). Enzymes oxidizing ethene to either 1,2-epoxyethane or carbon dioxyde and water have been discovered in plant tissue (Beyer, 1984).

Possible functions of the ability of plants to oxidize ethene are (i) formation of 1,2-epoxyethane which synergizes with ethene and (ii) regulation of the internal ethene concentration. In Vicia, the enzymes involved were characterized as mono-oxygenases requiring oxygen and NADPH for activity (Smith et al, 1985).

Metabolism of ethene by several plant tissues has been extensively demonstrated. In tissue of Vicia faba cotyledons it was shown that propene and allene were also oxidized, whereas saturated hydrocarbons like methane and ethane were not metabolized (Abeles, 1984).

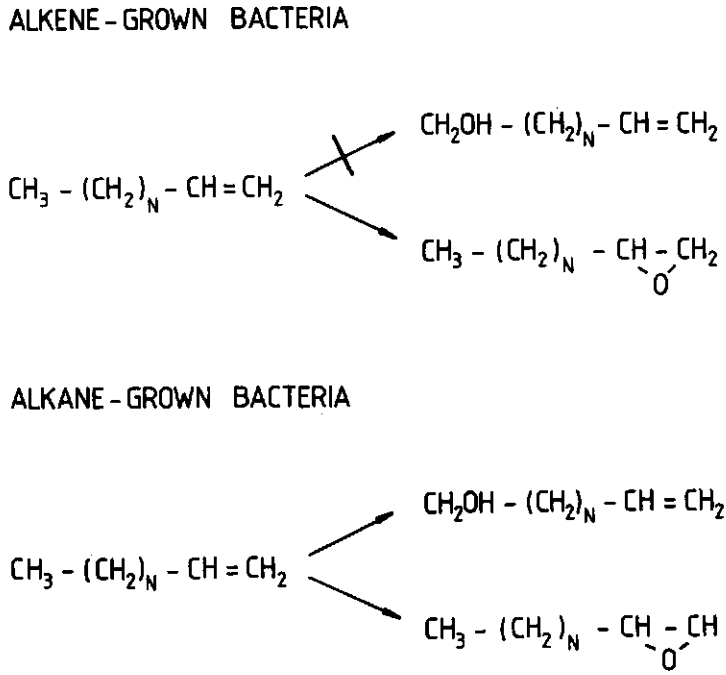

Figure 2. Initial oxidation reactions towards gaseous hydrocarbons by alkene- and alkane-grown bacteria

Vertebrates

Detoxification of organic compounds and excretion of products of their metabolism are essential for many living organisms.

Transformation of foreign compounds in vertebrates occurs mainly in the liver, especially in the smooth endoplasmatic recticulum (microsomes). Transformations such as hydrolysis, hydroxylation and epoxidation often result in an increase of polarity and therefore water solubility of the compounds involved. Unsaturated hydrocarbons may be oxidized to their respective epoxyalkanes and the enzymes involved are P450 mono-oxygenases. Epoxidation of a variety of unsaturated hydrocarbons such as ethene, 1,3-butadiene and isoprene by microsomal mono-oxygenase has been demonstrated (Ehrenberg et al, 1977; Malvoisin and Roberfroid, 1982; Filser and Bolt, 1983; Del Monte et al, 1985).

POSSIBLE APPLICATIONS OF HYDROCARBON-UTILIZING BACTERIA

Alkane-utilizing micro-organisms have been considered excellent candidates for the production of single-cell protein using cheap alkanes as growth substrates (Hamer and Harrison, 1980). More recently, the discovery of the extensive range of transformations catalysed by methane-grown bacteria has opened up the possibility of their use as biocatalyst and several patents have been filed on this subject. Knowledge of a similar potential of alkene-utilizing bacteria is still relatively scarce, but these organisms seem promising in (i) the production of chiral epoxides (Habets-Crützen et al, 1985) and (ii) in the removal of harmful alkenes from polluted air (Hartmans et al, 1985).

(i) Production of chiral epoxides

1,2-epoxyalkanes are produced in large amounts in the chemical industry because of their potential to serve as building blocks for chemical synthesis. 1,2-Epoxyethane and 1,2-epoxypropane constitute the two most important commercially available epoxides. Currently, production of bulk petrochemicals like epoxyalkanes by biotechnological means clearly has many disadvantages as compared to the existing chemical processes. However, the biotechnological production of chiral epoxyalkanes may have commercial potential if enantiomerically pure epoxides could be produced, these could form a starting point of stereochemical syntheses. It has already been shown that some alkene-utilizing bacteria are able to form epoxides with one enantiomer in high amounts (Habets-Crützen et al, 1985).

(ii) Removal of alkenes from gas phases

Unsaturated hydrocarbons like 1,3-butadiene, isoprene and especially some chlorinated alkenes like vinylchloride are potential carcinogens (De Meester et al, 1978; Infante, 1981;

Gervasi et al, 1985) These hazardous compounds may therefore not be released in the environment. Several chemical and/or physical methods have been developed to remove these hazardous compounds. Gaseous organic pollutants may also be eliminated from waste gases using a biological filter bed (gas/solid bioreactor). Such a filter bed could consist of an appropriate filling material on which micro-organisms are present or have been immobilized and thus most organic compounds may be oxidized to water and carbon dioxyde and, in case of chlorinated hydrocarbons, also to hydrochloric acid.

Many odourous compounds are perceptible in very low concentrations and some odour problems have been solved successfully with biological filter beds. In general, biological filters removing other pollutants like unsaturated hydrocarbons can only compete with chemical/physical methods when the concentrations in the waste gas are also in the range of a few volumes per million (Don 1983).

OUTLINE OF THE PRESENT INVESTIGATION

The aim of this investigation was to elucidate the role of alkene-utilizing micro-organisms in the oxidation of gaseous and volatile hydrocarbons. Furthermore, potential applications of alkene-utilizing bacteria e.g. production of chiral epoxyalkanes have been investigated and discussed.

Until now only Mycobacterium spp. and Nocardia spp. were known as alkene-utilizing bacteria and a very limited number of gaseous alkenes as for instance the plant hormone ethene have been investigated for their degradability. A more extensive study of the bacteria that utilize gaseous and volatile alkenes as carbon and energy source and the microbial oxidation of alkenes is presented in chapters 2, 3 and 4.

A description of microbial growth on gaseous alkenes in chemostat cultures is presented in chapter 5. The capacity of washed cell suspensions of alkene-grown bacteria to oxidize alkenes to either carbon dioxide and water or epoxyalkanes is presented in chapters 6 and 7. Chapter 6 deals with the oxidation of alkenes by alkene-grown Xanthobacter whereas chapter 7 gives a general discussion of gaseous hydrocarbon utilization and oxidation by selected alkene-utilizing bacteria.

Chapters 8, 9 and 10 deal with the behaviour of alkene-utilizing bacteria in gas/solid bioreactors (biological filter beds). The objectives of these studies were either to produce toxic epoxyalkanes or to oxidize ethene which is present in very low concentrations in the air in warehouses used for storage of fruit and vegetables.

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CHAPTER 2

ISOLATION AND CHARACTERIZATION OF ALKENE-UTILIZING XANTHOBACTER spp.

C.G. van Ginkel and J.A.M. de Bont

SUMMARY

Yellow-pigmented bacteria showing typical characteristics of Xanthobacter spp. were isolated from enrichments with propene and 1-butene, using classical techniques. The generation time for growth on propene and 1-butene of these bacteria ranged from 5 to 7 hours. A NADH-dependent mono-oxygenase was identified in cell-free extract of Xanthobacter Py2. This mono-oxygenase was not influenced by potential inhibitors tested indicating that propene mono-oxygenase is different from other hydrocarbon mono-oxygenases described until now. Nitrogenase activity could be measured using the acetylene reduction assay with propene as energy source, because acetylene did not inhibit the mono-oxygenase activity.

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INTRODUCTION

The production of 1,2-epoxyalkanes from alkenes in biotechnological processes has been studied with several micro-organisms including methane-utilizing bacteria (Higgins et al. 1979, 1980, Hou et al. 1979), propane- and butane-utilizing micro-organisms (Hou et al. 1983, Patel et al. 1983), heptane-utilizing Pseudomonas (van der Linden 1963) and the octane-utilizing Pseudomonas oleovorans (de Smet et al. 1981, 1983). The formation of 1,2-epoxyalkanes by these alkane-grown bacteria is due to the non-specific oxidation of alkenes by the alkane-hydroxylases. The formation of 1,2-epoxyalkanes has also been studied with alkene-utilizing bacteria like Nocardia corallina (Furahashi et al. 1981) and ethene-utilizing Mycobacterium strains (Habets-Crützen et al. 1984). Growth of micro-organisms on propene was first reported by Cerniglia et al. (1976) and later by de Bont et al. (1980). Mycobacterium PY1 isolated by de Bont et al. (1980) oxidized propene via a mono-oxygenase to 1,2-epoxypropane and this specific alkene-mono-oxygenase was also involved in the formation of 1,2-epoxyethane (de Bont et al. 1983). The alkene-utilizing Mycobacterium strains isolated until now are relatively slow growing. While trying to isolate faster growing micro-organisms on propene and 1-butene, we repeatedly isolated Xanthobacter spp.

In this report we describe the isolation and characterization of these alkene-utilizing Xanthobacter spp.

MATERIALS AND METHODS

Chemicals. Gaseous alkenes, ethyne and 1,2-epoxyethane were obtained from Hoek Loos, Amsterdam, The Netherlands. NADH and NADPH were purchased from Boehringher, Mannheim, West-Germany. All other chemicals were obtained from Janssen Chimica, Beerse, Belgium.

Micro-organisms. The Xanthobacter strains were isolated by methods described previously (Wiegant and de Bont 1980) except that ethene was replaced by higher concentrations (5%) of propene or 1-butene. Xanthobacter autotrophicus strain 7C and strain JW33 were obtained from Deutsche Sammlung von Mikroorganismen (DSM), Göttingen, FRG.

Maintenance and cultivation of the micro-organisms. The bacteria were maintained on yeast/glucose slopes. The micro-organisms were cultivated in mineral medium supplemented with the appropriate gaseous alkenes as described by Wiegant and de Bont (1980). The gas phase contained only 10 % (v/v) oxygen when the organisms were grown under nitrogen-fixing conditions in which case $(NH_4)_2SO_4$ and NH_4Cl in the medium were replaced by $MgSO_4$ and NaCl, respectively. Growth on other carbon and energy sources and the

determination of the doubling time has been described by Habets-Crützen et al. (1984).

Analyses. Gaseous alkenes were determined as described by de Bont et al. (1979). Ethene concentrations were measured during the acetylene reduction test in the same way as otherwise except that the oven temperature was 60°C instead of 180°C. Protein concentrations were determined as described by Habets-Crützen et al. (1984). The detection of mycolic acids was determined by thin layer chromatographic analysis of whole organisms methanolysates as described by Minnikin et al. (1975). GC content was determined as described by Mandel and Marmur (1968) and poly-β-hydroxybutyric acid was determined by the method described by Jüttner et al. (1975).

Preparation of washed cell suspensions and cell-free extracts. The preparation of washed cell suspensions and cell-free extracts has been described previously (de Bont and Harder 1978, de Bont et al. 1979). Cell-free extract was dialysed by eluating the extract over a G-25 Sephadex column.

Determination of kinetic constants. A washed cell suspension (0.5 mg protein) of propene-grown cells was incubated in screw-cap bottles in a shaking bath at 30° C. To measure Michaelis Menten constants, the reaction was started by injecting 500 ppm propene in the gas phase. Samples from the gas phase were withdrawn at regular intervals for gas chromatographic analysis. Michaelis Menten constants were calculated by fitting the integrated Michaelis Menten equation to the measured concentration-time data by means of a computer program.

Acetylene reduction test. Cells of a culture grown in a nitrogen-free medium under reduced oxygen tension were injected directly in Hungate tubes containing the appropriate gaseous mixtures. The tubes were incubated in a shaking bath at 30°C. The gas phase was analysed for ethene at regular intervals.

Propene mono-oxygenase. Oxidation of propene by cell-free extracts was carried out as described by de Bont and Harder (1978). Inhibitors were injected just before starting the reaction with propene.

RESULTS

Isolation

Bacteria able to grow on either propene or 1-butene were enriched by incubating various soil and water samples in an atmosphere of 5 percent alkene in air. Organisms were isolated from such enrichments in the presence of either propene or 1-butene by subculturing in a liquid mineral medium and by streaking cells to purity on agar plates of mineral medium. In this way, six strains were isolated from enrichments in the presence of propene (Py2, Py3, Py7, Py10, Py11, Py17) and one strain was isolated in the presence of 1-butene (By2). The specific growth rate of these strains on propene and 1-butene ranged from 0.14 to 0.10 hour^{-1} which is about 5 times faster

than the specific growth rate of Mycobacterium Py1 on 2% propene in air.

Table 1. Kinetic constants for propene of Mycobacterium Py1 and two Xanthobacter strains.

Bacteria	V_{max} nmol min^{-1} (mg protein)$^{-1}$	K_m 1) vpm
Mycobacterium Py1	15	100
Xanthobacter Py2	70	280
Xanthobacter Py10	65	230

1) K_m has been related to the concentration in the gas phase.

Mycobacterium Py1 was originally isolated using low concentrations of propene, while the newly isolated strains were obtained using high concentrations of alkene. The isolation of the new strains might therefore be a consequence of different affinities towards alkenes. Therefore, the K_m and V_m for propene of washed cell suspensions of strain Py2 and Mycobacterium Py1 were measured (Table 1).

Characterization

The seven strains were all immotile, irregularly-shaped rods which divided by snapping. The organisms were pleomorph as illustrated by Fig. 1 showing strain Py2 grown on propene (A), succinate (B) and 1-propanol (C), respectively. They formed round, slimy, yellow colonies when plated on yeast/glucose medium. Slime-free mutants could be isolated easily from a carbon limited chemostat culture. The strains were all able to use other substrates for growth as for instance H_2/CO_2, methanol, ethanol, 1-propanol, propanal, acetone, 2-propanol, 1,2-propanediol, propionate, 1,2-epoxypropane, pyruvate, 1,2-epoxybutane, 1-butanol, 1,2-butanediol, glucose, fructose and glutamate. These strains were also able to grow on ethene, albeit with doubling times of 20 hours or more. No growth was observed on ethane, propane, butane or galactose. The strains isolated could utilize NH_4^+ or NO_3^- as nitrogen source and fixed atmospheric nitrogen at reduced oxygen levels. No mycolic acid could be detected in either strain Py2 and strain Py10. It was shown that poly-β-hydroxybutyric acid and catalase were present in strain Py2 and strain Py10. The GC content of Xanthobacter Py2 was 70.0%. The physiological and morphological data of strain Py2 and Xanthobacter autotrophicus are summurized in Table 2. On basis of these properties the bacteria were assigned to the

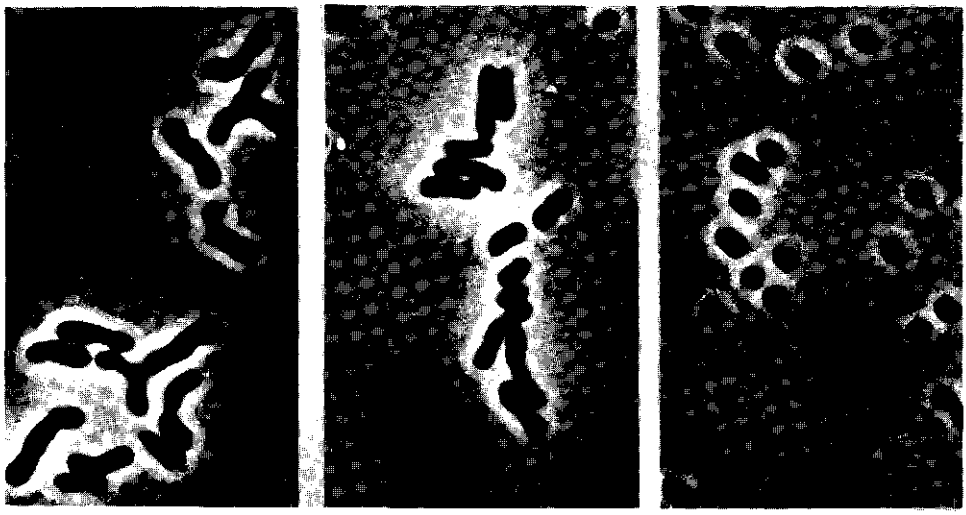

Figure 1. Phase-contrast light micrograph of Xanthobacter Py2 grown on succinate (A), propene (B) and 1-propanol (C). The bar in the figures represents 1 μm.

Table 2. Characteristics of Xanthobacter Py2 and Xanthobacter JW 33.

Characters	Xanthobacter autotrophicus JW33	Xanthobacter Py2
G+C content	68.9	70
Cyst formation	-	-
Storage material	PHB	PHB
Refractile bodies	+	+
Slime production	+	+
Colony color	yellow	yellow
Cell size	0.4-1.0	0.4-1.0
Pleomorphism	+	+
Motility	-	-
Catalase production	+	+
Gram reaction	variable	variable

genus Xanthobacter. For comparison Xanthobacter autotrophicus strains 7C and JW33 were also tested for growth on alkenes but

these bacteria were not able to grow on either ethene, propene or 1-butene.

Acetylene reduction with propene

The reduction of acetylene to ethene is a measure for the rate of nitrogen fixation by bacteria. Acetylene reduction by Xanthobacter Py2 was only possible at reduced oxygen levels. Fig. 2 shows that 20 percent of oxygen in the gas phase totally inhibited acetylene reduction. Acetylene reduction occurred with propene as energy source and the rate of acetylene reduction was not influenced by the propene concentration (Fig. 2).

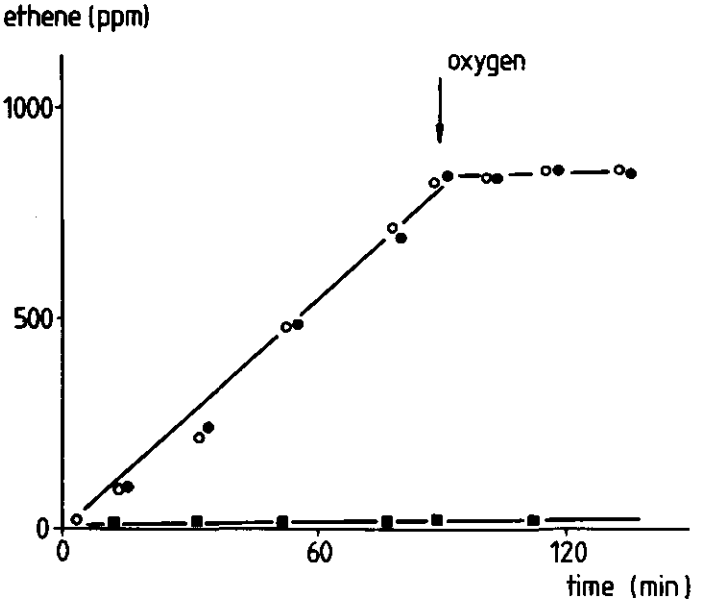

Figure 2. Acetylene reduction by propene-grown Xanthobacter Py2 as influenced by propene and oxygen. The oxygen concentration in the gas phase (5%) was increased to 20 percent after 90 minutes of incubation. The propene concentration in the gas phase was 0% (■), 5% (○) and 19% (●).

Propene mono-oxygenase

The disappearance of propene was measured in vitro to establish the nature of the enzyme involved in the conversion of propene. The oxidation of propene by a cell-free extract was supported by NADH in the presence of oxygen indicating that the enzyme involved is a mono-oxygenase (Fig. 3). NADH could be replaced by NADPH but not by other electron donors like $FADH_2$

or ascorbic acid. Some potential inhibitors of hydrocarbon mono-oxygenases were tested but only in few cases an effect on propene oxidation was recorded (Table 3). CN^- at higher concentrations, and allylthiourea inhibited the propene mono-oxygenase for only 30 percent and also ethyne inhibited the mono-oxygenase only slightly.

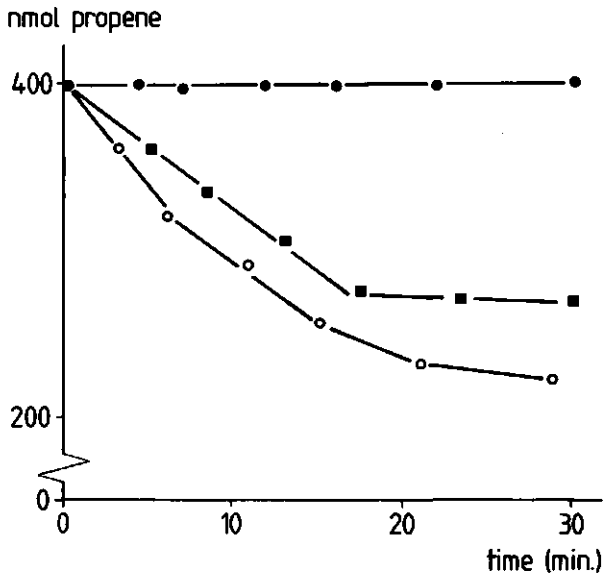

Figure 3. Oxidation of propene by dialysed cell-free extracts (13 mg protein) of Xanthobacter Py2 grown on propene without cofactor (●) and in the presence of NADH (○) or NADPH (■).

DISCUSSION

Bacteria growing on either ethene, propene or 1-butene have been described by several authors (de Bont 1976, de Bont et al. 1980, Cerniglia et al. 1976, Heyer 1976) but so far only strains belonging to the genus Mycobacterium and an unidentified strain were isolated on these alkenes. The isolation of these Mycobacteria was generally achieved by methods involving low concentrations of alkene in the gas phase. We have now used higher concentrations of propene and 1-butene in the gas phase (5 percent) while enriching for alkene-utilizers resulting in the isolation of faster growing Xanthobacter spp. It seems that

so far only the slower growing Mycobacterium were isolated on propene because these organisms have a higher affinity towards the alkene than the Xanthobacter strains. An indication that Mycobacteria have a higher affinity towards propene than the newly isolated Xanthobacter is the difference in the K_m for propene of Xanthobacter Py2 and Mycobacterium Py1.

Table 3. Effect of potential inhibitors of propene mono-oxygenase activity on propene oxidation by cell-free extract of Xanthobacter Py2.

Inhibitor	Concentration	Relative activity (%)
None	-	100
Imidazole	0.1 mM	97
2-Mercaptoethanol	0.1 mM	118
CN	1 mM	67
CN	0.1 mM	81
CN	0.01 mM	101
8-Hydroxyquinoline	1 mM	99
8-Hydroxyquinoline	0.1 mM	126
Thiourea	0.1 mM	126
Allylthiourea	0.1 mM	77
CO	10 %	105
Ethyne	4 %	72

The carbohydrate utilization, colony and cell morphology of the newly isolated strains are in agreement with previous published data for Xanthobacter spp. The pleomorphism of the strains, along with the copious slime production, are also similar to other Xanthobacter spp. Also, the isolation of slime-free mutants of Xanthobacter spp. has been described by several authors. Xanthobacter Py2 grown on succinate is showing a typical branched cell formation and cells grown on 1-propanol show coccoid cell formation as described by Wiegel et al. (1978). Wiegel et al. (1978) also described that their Xanthobacter did not possess mycolic acids. Mycolic acids could not be detected in our strains, while a multi spot pattern was shown on the thin layer chromatography plates of the propene-utilizing Mycobacterium Py1 (de Bont et al. 1980). The GC content of Xanthobacter Py2 is in agreement with data published by Wiegel et al. Growth of Xanthobacter on alkenes is not a general property of these bacteria since the type strains JW33 and 7C did not utilize these compounds.

The alkene-utilizing Xanthobacter are all able to fix atmos-

pheric nitrogen at reduced oxygen levels. Acetylene was also reduced to ethene with propene as energy source which is surprising because other hydrocarbon-utilizing bacteria as for instance methane-oxidizing bacteria cannot reduce acetylene. The inability to detect nitrogen fixation in methane-oxidizing bacteria with the acetylene reduction test was caused by the inhibition of methane mono-oxygenase by acetylene (de Bont and Mulder 1976).

The propene mono-oxygenase of Xanthobacter Py2 resembles the alkene mono-oxygenase of Mycobacterium Py1 and Mycobacterium E20 in that it only oxidizes unsaturated hydrocarbon bonds to 1,2-epoxyalkanes and does not hydroxylate alkanes, and in that only NADH and NADPH acted as electron donors. To preliminary characterize the enzyme, some potential inhibitors of hydrocarbon mono-oxygenases were tested. Acetylene, an inhibitor of other hydrocarbon mono-oxygenases did not inhibit the propene mono-oxygenase, and furthermore other potential inhibitors did not act on the mono-oxygenase with an exception for CN^- at higher concentrations. This suggests that the propene mono-oxygenase is different from other hydrocarbon mono-oxygenases described to date (Cardini and Jurtshuk 1970, Colby and Dalton 1976, McKenna and Coon 1970, Tonge et al. 1977). Further research with the alkene-utilizing strains will concentrate on epoxide formation by these organisms.

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CHAPTER 3

MICROBIAL OXIDATION OF ISOPRENE, A BIOGENIC FOLIAGE VOLATILE AND OF 1,3-BUTADIENE, AN ANTHROPOGENIC GAS

C.G van Ginkel, E. de Jong, J.W.R Tilanus and J.A.M. de Bont

SUMMARY

Nocardia strains that were able to degrade isoprene were isolated from several locations using enrichment cultures with isoprene or 1,3-butadiene as the sole carbon and energy source. Specific growth rates of representative isolates on isoprene and 1,3-butadiene ranged from 0.05 to 0.2 h^{-1}. The initial oxygenation of both 1,3-butadiene and isoprene was mediated by mono-oxygenases which converted these alkadienes into their respective epoxyalkanes.

Submitted for publication

INTRODUCTION

Isoprene (2-methyl-1,3-butadiene) is a naturally occuring compound which is excreted by leaves of higher plants (1). Isoprene has also been identified as a component of roasted coffee and is the main component of unsaturated gaseous hydrocarbons in tabacco smoke (2,3). In industry, isoprene is extensively used, primarily as a monomer for the synthesis of plastics and rubber. The analogous compound 1,3-butadiene, to our knowledge, does not occur naturally but it is also manufactured in large amounts and serves in the same applications as does isoprene.

It has been demonstrated that both 1,3-butadiene and isoprene are epoxidized by microsomes of rat liver (4,5). Epoxides of 1,3-butadiene and isoprene are mutagenic and/or carcinogenic (6,7).

Surprisingly, there are only few published data on the biotransformation of isoprene by micro-organisms. Propene-grown Xanthobacter spp. and methanotrophs were able to oxidize isoprene but isoprene did not serve as a sole carbon and energy source for growth (8,9). Watkinson and Sommerville (10) have isolated an organism tentatively identified as a Nocardia sp., capable of growth on 1,3-butadiene and some 1-alkene-utilizing Xanthobacter spp. were also able to use 1,3-butadiene as a carbon and energy source (9). Moreover, 1,3-butadiene is oxidized by all alkane-grown bacteria tested (11,12,13).

We now have isolated several bacteria that are able to grow on isoprene and 1,3-butadiene as a sole source of carbon and energy.

MATERIALS AND METHODS

Chemicals. Gaseous alkenes were obtained from Hoek Loos, Amsterdam, The Netherlands. NADH was purchased from Boehringer, Mannheim, FRG. All other chemicals were obtained from Janssen Chimica, Beerse Belgium. Epoxides from isoprene were prepared as previously described by Imata and Ziffer (14). The three epoxides synthesized were identified by GC-MS.

Micro-organisms. The Nocardia strains were isolated by methods described previously (15), except that ethene was replaced by 1,3-butadiene or isoprene. Nocardia vacinni was a kind gift of Prof. D. Jones, Dept. of Microbiology, University of Leicester, UK.

Maintenance and cultivation of micro-organisms. The bacteria were maintained on yeast extract/glucose slopes. The organisms were cultivated in a mineral salts medium supplemented with the appropriate alkadienes as described by Wiegant and de Bont (16). Growth on other carbon and energy sources and the determination of the doubling times was as described by

Habets-Crützen et al. (17).

Analyses. Gaseous alkadienes and epoxides were determined as described by de Bont et al. (18). Protein concentrations were determined as described by Habets-Crützen et al (17). The detection of mycolic acids was determined by thin layer chromatography of whole organism methanolysates as described by Minnikin et al. (19). Dissolved O_2 concentrations were measured at 30 ° C with a Yellow Springs Instruments Co. Model 53 monitor equipped with a polarographic sensor.

Preparation of washed cell suspensions and cell-free extracts. The preparation of washed cell supensions and cell-free extracts has been described previously (18,20). Cofactors were removed from cell-free extracts by eluating the extract over a G-25 Sephadex column.

Oxidation of gaseous hydrocarbons and inhibition studies. Oxidation of isoprene or 1,3-butadiene by washed cell suspensions and cell-free extracts was carried out as described by Habets-Crützen et al. (17). Accumulation of epoxides from alkadienes was studied in the presence of either 100 mM 1,2-epoxypropane or 1,2-epoxybutane.

RESULTS AND DISCUSSION

An indication of the distribution of isoprene-utilizing micro-organisms in soil is the capacity of various soil samples to take up this compound from the gas phase. Therefore, soil samples from ten different sites were placed in screw-cap bottles and isoprene was injected into the gas phase of the bottles. The rate of isoprene disappearance from the gas atmosphere in the bottles varied depending on the soil sample but within a week in all bottles isoprene was completely consumed. Sterilized soil in control bottles did not consume isoprene even after four weeks of incubation, demonstrating the involvement of micro-organisms in the removal of isoprene. Fig. 1 shows the uptake of isoprene by sandy soil as influenced by different initial concentrations of the chemical. As evidenced from an increase in the isoprene uptake rate with time, isoprene-degrading micro-organisms either grew in soil or the enzymes responsible for isoprene degradation were induced. Similar experiments were carried out with the gaseous alkenes 1,3-butadiene and ethene (data not shown). 1,3-Butadiene was consumed by soil bacteria at the same rate as isoprene, whereas the uptake rate of ethene, a naturally occuring plant hormone, by soils was very low as compared to alkadiene uptake rates (15). Comparable uptake rates of both alkadienes suggest that the same bacteria present in soil may be involved in metabolizing both hydrocarbons.

In order to isolate these isoprene- and 1,3-butadiene-utilizing organisms, soil samples from various locations were

incubated in a mineral salts medium with isoprene or 1,3-butadiene as sole carbon and energy source. Within a few days all cultures showed growth and after repeated subculture the bacteria were streaked to purity. The isolation procedure resulted in six 1,3-butadiene- and isoprene-utilizing bacteria which strongly resemble each other. Strains BT1, BT2, BT3 were iso-

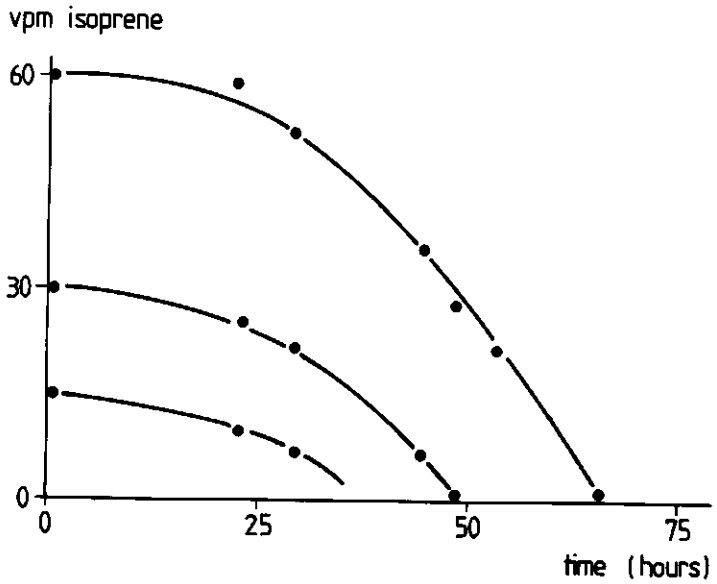

Figure 1. Uptake of isoprene by sandy forrest soil as influenced by various initial concentrations of isoprene. 100 Cm^3 screw-cap bottles contained 10 g of soil.

lated on 1,3-butadiene and strains IP1, IP2, IP3 were isolated on isoprene as carbon and energy source. Attempts were carried out to isolate isoprene-utilizing micro-organisms using leaves of various higher plants as inoculum, but positive enrichment cultures were not obtained. The hypothesis of Rasmussen and Hutton (21) that micro-organisms growing on leaves and barks which utilize volatile hydrocarbons may act as a biological sink was not confirmed by our results. The ability of soil bacteria to remove isoprene almost immediately at very low concentrations is impressive (Fig. 1). Rasmussen (1) reported that the isoprene concentration in air over mango leaves was 0.6 ppb, but it remains uncertain whether isoprene-utilizing soil bacteria are able to remove isoprene at such low concentrations from the atmosphere. The precise chemical or biological fate of

isoprene has to be elucidated by further studies.

All isolates obtained were Gram-positive, non-motile pink pigmented bacteria. The strains were pleomorph as illustrated by Fig. 2 showing strain IP1 grown on 1,3-butadiene (A), isoprene (B) and succinate (C), respectively. The bacteria formed convex circular colonies when plated on mineral salts medium

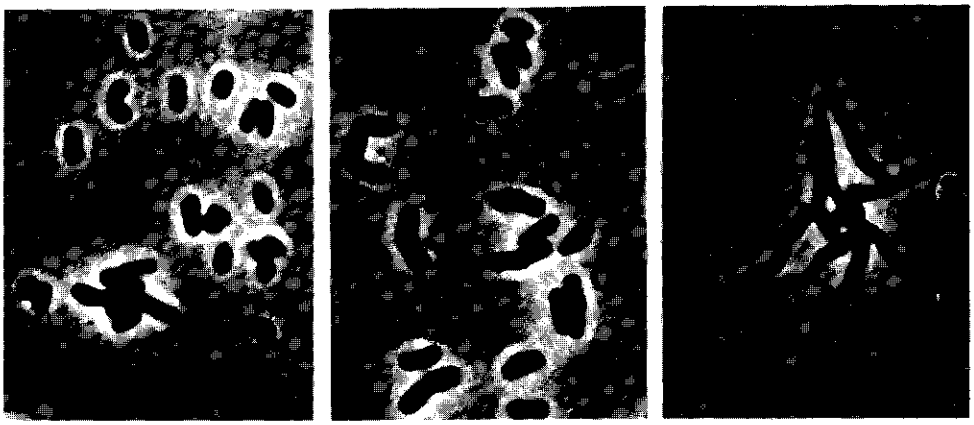

Figure 2. Phase-contrast light micrograph of Nocardia IP1 grown 1,3-butadiene (A), isoprene (B) and succinate (C). The bar represents 1 µm.

with alkadienes as carbon and energy source in the gas phase. Upon mycolic acid analysis, the methanolysate of the isolated strains showed the same pattern as Nocardia vaccinii. The strains were not able to reduce nitrate to nitrite. On the basis of these properties, the bacteria were tentatively assigned to the genus Nocardia. The bacteria were able to grow on several substrates including ethanol, 1-propanol, 2-propanol, 1,2-propanediol, glucose, succinate, phenol and dextrin. More interesting was the capacity of all strains to grow on 1,3-butadiene, isoprene and butane, whereas 1-alkenes and other gaseous alkanes did not support growth. The specific growth rate on isoprene or 1,3-butadiene of the Nocardia spp. ranged from 0.05 to 0.2 h^{-1}. The resemblance between the strains isolated on 1,3-butadiene and isoprene suggests a connection between metabolic pathways of both alkadienes. The capacity to degrade anthropogenic 1,3-butadiene by these Nocardia spp. may

have developed from the potential of these organisms to metabolize isoprene.

The initial oxidation of 1,3-butadiene and isoprene is mediated by a mono-oxygenase as shown in cell-free extracts of alkadiene-grown Nocardia IP1 which were only capable of oxidizing these hydrocarbons in the presence of NADH and oxygen. By analogy to other alkene-degrading bacteria and alkadiene oxidation in microsomes, the first intermediate in the degradation pathways of 1,3-butadiene and isoprene would be epoxyalkanes (4,5,18,22). For this reason, experiments were carried out to detect intermediates of 1,3-butadiene and isoprene oxidation in washed cell suspensions of 1,3-butadiene- and isoprene-grown Nocardia IP1 using 1,2-epoxyalkanes as competitive inhibitors. From 1,3-butadiene, the corresponding 1,2-epoxy-3-butene was recovered, whereas from isoprene three epoxides were recovered, demonstrating that epoxidation reactions are very likely. These products of alkadiene oxidation were identified by gaschromatography retention time comparison and cochromatography with the authentic epoxides. Moreover, washed cell suspensions of 1,3-butadiene-grown Nocardia IP1 excreted trace amounts of 1,2-epoxy-3-butene when incubated in the presence of 1,3-butadiene, indicating that this epoxide is the first intermediate in the degradation pathway of 1,3-butadiene. 3,4-Epoxy-3-methyl-1-butene is probably the first intermediate in the degradation pathway of isoprene because this compound and the diepoxide were detected during the oxidation of isoprene by washed cell suspensions of isoprene-grown Nocardia IP1. However, the diepoxide formed was not metabolized by washed cell suspensions of isoprene-grown Nocardia IP1. Furthermore, washed cell suspensions of alkadiene-grown bacteria did oxidize the monoepoxide of 1,3-butadiene whereas the diepoxide did not enhance the oxygen uptake rate. On basis of simultaneous adaption studies, Watkinson and Sommerville (10) also suggested that 1,2-epoxy-3-butene is an intermediate in the 1,3-butadiene catabolism. The further metabolism of both alkadienes has not been elucidated.

Acknowledgement; We are indebted to Dr M.A. Posthumus for performing the mass spectrum analyses of the epoxides.

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CHAPTER 4

METABOLISM OF TRANS-2-BUTENE AND BUTANE IN NOCARDIA TB1

C.G. van Ginkel, H.G.J. Welten, S. Hartmans and J.A.M. de Bont

SUMMARY

A bacterium capable of growth on trans-2-butene was isolated from soil and identified as a Nocardia sp. The isolate TB1 also grew on propane, butane, pentane and hexane; gaseous and volatile 1-alkenes did not support growth. Intact cells grown on trans-2-butene or butane oxidized all n-alkanes, 1-alkenes, 2-alkenes and alcohols tested. Simultaneous adaptation studies, inhibitor experiments and measurements of enzyme activities in crude extracts indicated a degradative route of trans-2-butene via crotonic acid and of butane via butyric acid. The key enzymes in the proposed pathways were induced by growth on either trans-2-butene or butane. The induction of these enzymes and the substrate specificities of the enzymes suggest a relation between trans-2-butene and butane degradation.

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INTRODUCTION

Micro-organisms attack a wide range of both saturated and unsaturated hydrocarbons (Klug & Markovetz, 1971) and also the lower, gaseous compounds are readily degraded as originally shown by Tautz & Donath (1930). Bacteria utilizing saturated gaseous hydrocarbons mainly belong to the genera Mycobacterium, Nocardia, Corynebacterium, Brevibacterium and Pseudomonas (Klug & Markovetz, 1971), and most of these bacteria grow well on ethane, propane or butane (Hou et al., 1983; Patel et al., 1983; Philips & Perry, 1974). In general, the pathway of alkane degradation proceeds via terminal hydroxylation of the alkane, yielding the corresponding primary alcohol (Klug & Markovetz, 1971) but subterminal oxidations have been demonstrated as well (Vestal & Perry, 1969).

The ability of micro-organisms to grow on gaseous alkenes has been described for ethene (de Bont, 1976; Heyer, 1976), propene and 1-butene (de Bont et al., 1980; Cerniglia et al., 1976; van Ginkel & de Bont, 1986) and for 1,3-butadiene (Watkinson & Sommerville, 1976). The first intermediate in the metabolism of gaseous 1-alkenes is the corresponding 1,2-epoxyalkane formed by a mono-oxygenase (de Bont et al., 1979; 1983). The further metabolism of 1,2-epoxyethane proceeds via acetyl-CoA (de Bont & Harder, 1978), and the metabolism of 1,2-epoxypropane presumably via a C_2 + C_1 cleavage (Cerniglia et al., 1976).

A major difference between alkane- and alkene-grown bacteria is the substrate specificity towards gaseous hydrocarbons of the mono-oxygenases responsible for the initial oxygenation. Alkane mono-oxygenases from alkane-utilizers are able to oxidize both alkanes and 1-alkenes (Higgins et al., 1980; Hou et al., 1979; Hou et al., 1983; Patel et al., 1983) whereas the alkene mono-oxygenases are only able to epoxidate alkenes (Habets-Crützen et al., 1984; van Ginkel et al., 1986).

Knowledge on the metabolism of lower 2-alkenes, in contrast to the situation for both 1-alkenes and alkanes, is scarce. Fujii et al. (1985) reported a 2-butene-utilizing Mycobacterium which was used in a microbial screening test. We now have isolated a trans-2-butene-utilizing Nocardia which was also able to grow on butane. This investigation was initiated to determine the initial attack of trans-2-butene and in this paper we also report on the metabolism of trans-2-butene and of butane in Nocardia TB1.

MATERIALS AND METHODS

Chemicals. Gaseous alkenes and 1,2-epoxyethane were obtained from Hoek Loos, Amsterdam, The Netherlands. Trans-2-butene was obtained from Hicol bv, Oud-Beyerland, The Netherlands. NADH was purchased from Boehringer, Mannheim, West-Germany. All other chemicals were obtained from Janssen Chimica, Beerse, Belgium.

Micro-organisms. Nocardia TB1 was isolated by similar methods as described by de Bont (1976) except that ethene was replaced by trans-2-butene. Nocardia vaccinii was a gift of Prof. D. Jones. Dept. of Microbiology Leicester.

Cultivation of the micro-organism. The micro-organisms were cultivated in mineral salts medium supplemented with trans-2-butene or butane as described by Wiegant & de Bont (1980). Growth on different carbon sources was determined in 100 cm^3 Erlenmeyer flasks containing 10 cm^3 mineral salts medium supplemented with the appropriate carbon source. The concentration of the gaseous substrates supplied was about 5% (v/v) while the concentration of the other carbon sources was 0.2% (w/v).

Analyses. Determination of gaseous alkenes and 1,2-epoxyalkanes has been described by de Bont et al. (1979). CO_2 was measured as described by de Bont et al. (1983). Acids were measured with a Varian Aerograph series 2400 gaschromatograph fitted with a Chromosorb 101 (80 to 100 mesh) column; the column temperature was 190°C and the carrier gas was N_2 saturated with formic acid. TOC was determined with a Dohrmann type DC organic carbon analyser. Dissolved O_2 concentrations were measured at 30° C with a Yellow Springs Instrument Co. model 53 monitor equipped with a polarographic sensor. Protein concentrations of washed cell suspensions and cell-free extracts were determined as described by Habets-Crützen et al. (1984). Mycolic acids were determined as described by Minnikin et al. (1975).

Determination of the doubling times. This was done as described by Habets-Crützen et al.(1984).

Preparation of washed cell suspensions and cell- free extract. Preparation of washed cell suspensions has been described by de Bont et al. (1979).

Oxidation of hydrocarbons and epoxyalkanes. The oxidation of hydrocarbons and the excretion of epoxyalkanes by washed cell suspensions (2.5 cm^3) were measured by incubating the cells in a 50 mM phosphate buffer (pH = 7.0) at 30°C in Hungate tubes. The appropriate gas (0.075 cm^3) or 0.1 cm^3 of a 10 mM epoxyalkane solution were injected into the Hungate tubes. Samples from the gas phase were withdrawn at regular intervals and analysed for hydrocarbons and epoxyalkanes.

Enzyme activities. Hydrocarbon mono-oxygenases were measured as described by de Bont & Harder (1978) except that ethene was replaced by trans-2-butene or butane. For crotonic alcohol dehydrogenase, crotonic aldehyde dehydrogenase and butanal dehydrogenase, the reaction mixture contained (3 cm^3) 3 µmol alcohol or aldehyde, 3 µmol NAD and 130 µmol Tris/HCl buffer (pH = 8.5) and the change in A_{340} due to NAD reduction was

followed. Butanal reductase was assayed by following NADH oxidation. The reaction mixture (1 cm^3) contained 130 µmol phosphate buffer (pH = 7.0), 0.6 µmol NADH and the reaction was started by adding 3 µmol butanal. Thiokinases were assayed by following the formation of a hydroxamic acid complex which was measured spectrofotometrically at 540 nm. The reaction mixture (1 cm^3) contained 40 µmol Tris/HCl (pH = 7), 10 µmol $MgCl_2$, 2.5 µmol ATP, 0.5 µmol CoA, 200 µmol hydroxylamine, 10 µmol crotonic acid or butyric acid and the reaction was stopped after 30 minutes by adding 1 cm^3 of a solution of 12% 3 n HCl and 5% (v/v) TCA in 0.1 n HCl. The extinction coefficient used was 1.56 10^{-4} mol/dm^3 (Beinert et al., 1953). The following enzymes were assayed as previously described: thiolase (Senior & Dawes, 1973) and isocitrate lyase (Dixon & Kornberg, 1959).

CO_2 production from crotonic alcohol and 2,3-epoxybutane. Washed cell suspensions of trans-2-butene-grown cells were incubated in 50 cm^3 screw-cap bottles at 30 ° C with 0.4% trans-2-butene in the gas phase, 0.2 mM 2,3-epoxybutane or 0.2 mM crotonic alcohol. CO_2 production was followed by measuring the CO_2 concentration in the gas phase at regular intervals.

Inhibition studies. Washed cell suspensions of trans-2-butene-and butane-grown Nocardia TB1 were incubated at 30°C with 5 mM arsenite or 100 mM fluoroacetate in screw-cap bottles (50 cm^3). Trans-2-butene or butane was injected into the bottles and the change in the concentration of trans-2-butene or butane was measured. Any accumulation of acids was also measured by gas chromatography, using samples of the supernatant fluid collected after centrifugation. Washed cells of trans-2-butene-grown Nocardia TB1 were incubated at 30 ° C in 50 cm^3 screw-cap bottles. 2,3-Epoxybutane (2.5 µmol) was injected and the oxidation rate of the epoxide was measured. Inhibition of the oxidation rate by trans-2-butene was measured after 17 minutes by injecting various amounts of trans-2-butene in the screw-cap bottles.

RESULTS

Isolation and characterization

A bacterium capable of growth on trans-2-butene was enriched by incubating a sample of ditch water in the presence of trans-2-butene. From this enrichment a red-pigmented Gram-positive bacterium was isolated, which produce leathery, dry and crusty colonies when growing on a solid mineral salts medium with trans-2-butene in the gas phase as carbon and energy source. Aerial hyphea were not produced by this strain. The bacterium formed a branched mycelium typical of Nocardia sp. as illustrated by Fig. 1 showing the bacterium grown on succinate (A) or trans-2-butene (B), respectively. On mycolic acid analysis, the methanolysate of the isolated strain showed the same pattern as that of Nocardia vaccinii. The bacterium was able to grow on several substrates including phenol, dextran, acetate,

propionate, butyrate, ethanol, 1-propanol, 1-butanol, 2-butanol, glucose and H_2/CO_2, but not on methanol. The bacterium was able to reduce nitrate to nitrite. The isolate did not hydrolyse gelatine or xanthine. On the basis of the above characteristics the organism was tentatively classified as a Nocardia sp.

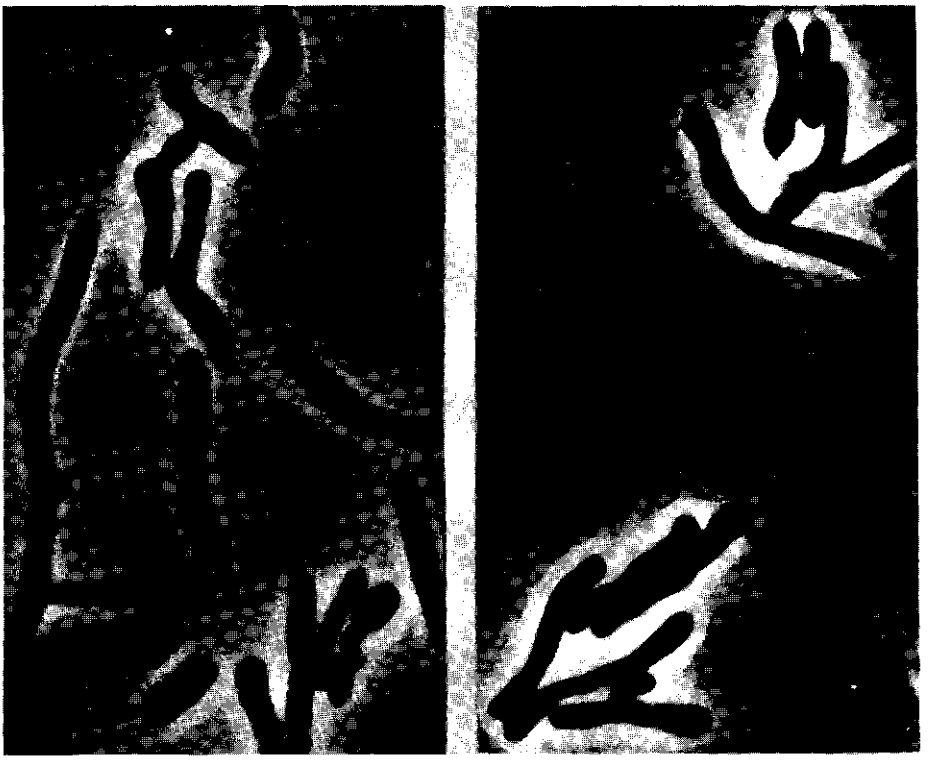

Figure 1. Phase-contrast light micrograph of Nocardia TB1 grown on succinate (A) and trans-2-butene (B). The bar represents 1.5 μm.

Nocardia TB1 is the first bacterium isolated on trans-2-butene and therefore it was of interest to determine which gaseous and volatile hydrocarbons could serve as a growth substrate. This strain was able to grow on the saturated hydrocarbons propane, butane, pentane, hexane and hexadecane but not on methane and ethane. The unsaturated hydrocarbons ethene, propene, 1-butene, 1,3-butadiene, 1-pentene and 1-hexene were no growth substrates. Growth on cis-2-butene was very slow. Culture doubling times of the isolated strain on butane and trans-2-butene were 6 and 30 h, respectively.

Oxidation of hydrocarbons by trans-2-butene- and butane-grown Nocardia TB1

Washed cell suspensions of trans-2-butene-grown Nocardia TB1 were able to oxidize all gaseous n-alkanes tested except methane (Table 1). Gaseous 1-alkenes were also oxidized, but the

Table 1. Oxidation of various hydrocarbons by washed cell suspensions of trans-2-butene- and butane-grown Nocardia TB1.

	Growth substrate	
Substrate	trans-2-Butene	Butane
	nmol min^{-1} (mg protein)$^{-1}$	
Methane	0	0
Ethane	0.7	1.8
Propane	1.5	3.7
Butane	2.5	4.9
trans-2-Butene	5.0	7.0
cis-2-Butene	5.7	8.0
1,3-Butadiene	3.4	7.0
Ethene	1.9	5.8
Propene	2.4	3.8
1-Butene	2.8	4.5

oxidizing activity leveled off after a short period of time when the corresponding 1,2-epoxyalkanes accumulated to a concentration of approximately 0.25 mM. From ethene and pro-pene, the 1,2-epoxyalkanes were formed in stoichiometric amounts but from 18 µmol 1-butene only 9 µmol 1,2-epoxybutane was formed. No epoxyalkanes accumulated from both trans-2-butene and cis-2-butene. Butane-grown cells oxidized the hydrocarbons tested, twice as fast as trans-2-butene-grown cells of Nocardia TB1 but at otherwise similar relative rates (Table 1) and the substrate oxidation rates also leveled off when 1,2-epoxyalkanes accumulated.

Inhibition studies with Nocardia TB1

Experiments to detect the products of butane and trans-2-butene oxidation with whole cells of Nocardia TB1, using different possible product analogues of the first oxidation reactions were performed.

Alcohols and epoxides that may compete for the active-site of the second enzyme in the degradative pathway of these hydrocar-

bons did not act as inhibitors. However, using the nonspecific

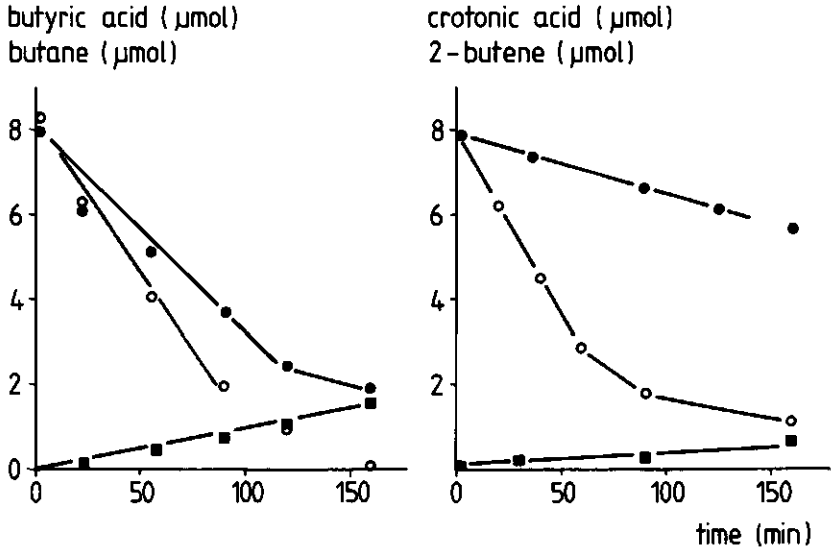

Figure 2. Effect of arsenite on the excretion of intermediates by washed cell suspensions of Nocardia TB1 grown on trans-2-butene (17 mg protein) or butane (14.5 mg protein). Closed symbols refer to incubations with inhibitor. trans- 2-butene (●), butane (●), crotonic acid (■) and butyric acid (■).

enzyme-inhibitor arsenite, it was possible to detect butyric acid and crotonic acid as possible intermediates of respectively butane and trans-2-butene metabolism (Fig. 2). Similar results were obtained using fluoroacetate as an inhibitor.

Substrate dependent oxygen uptake

Substrate dependent oxygen uptake was determined for trans-2-butene, butane-, and succinate-grown cells of Nocardia TB1 (Table 2). Crotonic alcohol and 2,3-epoxybutane stimulated the oxygen uptake by butane- trans-2-butene-grown cells whereas the oxygen uptake in succinate-grown cells was not stimulated by these substrates. Respiration of butane-grown cells was increased as compared to the endogenous respiration by 1-butanol and 2-butanol. 1-Butanol and 2-butanol did not or only slightly enhance the respiration rate of succinate-grown cells. Other possible intermediates in the degradative pathways of trans-2-butene and butane tested enhanced the oxygen uptake of trans-2-butene- as well as butane- and succinate-grown cells (Table 2).

Table 2. Increase in the rate of the oxygen uptake by washed cell suspensions of Nocardia TB1 grown on various substrates.

Substrate	Growth substrate		
	trans-2-Butene	Butane	Succinate
	nmol min-1 (mg protein)-1		
trans-2-Butene	64	112	0
cis-2-Butene	60	105	0
Crotonic alcohol	64	103	0
Crotonic aldehyde	80	102	12
Crotonic acid	63	63	19
Butane	46	53	0
1-Butanol	51	75	5
2-Butanol	19	28	0
Butanal	70	80	11
Butanone	12	38	5
Butyrate	31	52	19
3-Hydroxybutyrate	19	11	3
Acetate	57	48	32
2,3-Epoxybutane	18	16	0
Succinate	7	3	83

The endogenous rate of oxygen uptake by trans-2-butene-, butane- and succinate-grown cells was 13, 12 and 32 nmol min^{-1} (mg protein)$^{-1}$, respectively.

Enzyme activities

The nature of the enzyme system involved in the conversion of butane or trans-2-butene was investigated by measuring the disappearance of butane or trans-2-butene using cell-free extracts. Activity was only observed when NAD(P)H was included in the reaction mixture and when also molecular oxygen was present indicating that a mono-oxygenase is probably responsible for the oxidation of these hydrocarbons (Table 3).

A crotonic alcohol dehydrogenase and crotonic aldehyde dehydrogenase were induced after growth on trans-2-butene or butane and a 1-butanol dehydrogenase, measured as 1-butanal reductase, was also induced in butane- and trans-2-butene-grown cells. 2-Butanol dehydrogenase was also present but constitutive (Table 3). Thiokinases were induced in both trans-2-butene-, butane- and succinate-grown cells and the dependence of the enzyme for CoA and ATP was shown by omitting these cofactors from the assay mixture. Finally, it was shown that thiolase was pre-

sent in trans-2-butene-, butane- and succinate-grown cells (Table 3).

Evidence that butane is hydroxylated at the C_1 was also obtained by comparing isocitrate lyase levels in Nocardia TB1 after growth on various carbon sources (Table 4). Growth on butane and 1-butanol resulted in the induction of isocitrate lyase, whereas 2-butanol-grown cells contained no isocitrate lyase activity.

Table 3. Specific activities of enzymes in cell-free extracts of Nocardia TB1 grown on trans-2-butene, butane and succinate.

	Growth substrate		
Enzyme	t-2-Butene	Butane	Succinate
	nmol min^{-1} (mg protein)$^{-1}$		
trans-2-Butene monooxygenase (NADH)	2	2	0
Butane mono-oxygenase (NADH)	1	1	0
Crotonic alkohol dehydrogenase (NAD)	9	20	0
1-Butanol dehydrogenase (NAD)	0	0	0
Butanal reductase (NADH)	25	12	18
Crotonic aldehyde dehydrogenase (NAD)	3	5	0
Butanal dehydrogenase (NAD)	12	76	3
Crotonic acid thiokinase (ATP, CoA)	12	17	10
2-Butanol dehydrogenase (NAD)	5	5	4
Butyric acid thiokinase (ATP, CoA)	15	9	10
Thiolase	154	153	150

Table 4. Isocitrate lyase levels in cell-free extracts of Nocardia TB1 after growth on various substrates.

Growth substrate	Activity
	nmol min^{-1} (mg protein)$^{-1}$
Acetate	417
Propionate	3
Butyrate	220
Succinate	1
trans-2-Butene	110
Butane	137
1-Butanol	171
2-Butanol	1

The fate of trans-2,3-epoxybutane

Trans-2,3-epoxybutane was oxidized by butane- and trans-2-butene-grown Nocardia TB1 (Table 2). The oxidation of cis-2,3-epoxybutane was not investigated because this compound was not available. The trans-2,3-epoxybutane oxidation by a washed cell suspension of trans-2-butene-grown cells was inhibited by trans-2-butene, indicating that the mono-oxygenase present in Nocardia TB1 was probably responsible for the oxidation of trans-2,3-epoxybutane. Trans-2,3-epoxybutane was not oxidized to CO_2 because from this substrate hardly any additional CO_2 was formed as compared to the amount of CO_2 formed endogenously whereas washed cell suspensions of trans-2-butene-grown Nocardia TB1 produced twice the amount of CO_2 formed during the endogenous respiration when oxidizing trans-2-butene or crotonic alcohol (Fig 3). Organic carbon derived from an unknown oxidation product accumulated in the supernatant during 2,3-epoxybutane oxidation by washed cell suspensions of trans-2-butene-grown Nocardia TB1 whereas during growth on trans-2-butene, no organic carbon could be detected in the mineral salts medium.

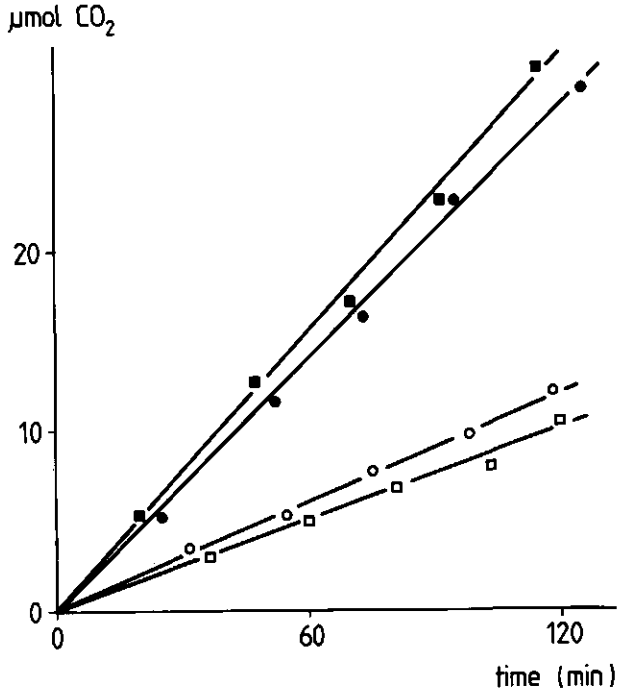

Figure 3. CO_2 production by a washed cell suspension of trans-2-butene-grown Nocardia TB1 (9 mg protein) in the absence (□) and the presence of trans-2-butene (●), crotonic alcohol (■) and 2,3-epoxybutane (○)

DISCUSSION

Nocardia TB1 was isolated with trans-2-butene as carbon and energy source and also grew on several saturated straight chain hydrocarbons but not on other alkenes. Micro-organisms that grow on both saturated and unsaturated gaseous hydrocarbons have been reported i.e. Mycobacterium E20 (de Bont et al., 1979), a Nocardia sp. (Fujii et al., 1985) and a "methanbacterium" (Tautz & Donath, 1930) but most hydrocarbon-utilizing bacteria cannot grow on both saturated and unsaturated hydrocarbons (Lukins & Foster, 1963; Habets-Crützen et al., 1984; van Ginkel et al., 1986). Both trans-2-butene- and butane-grown cells of Nocardia TB1 oxidized a wide range of hydrocarbons (Table 1). The differences in the rates of oxidation of trans-2-butene- and butane-grown cells were probably due to the specific growth rates of Nocardia TB1 on both substrates. The capacity to oxidize such a wide range of substrates is not unique. Methane-utilizers are also capable of oxidizing such a wide range of hydrocarbons (Hou et al., 1979; Higgins et al., 1980). Other alkane-utilizing micro-organisms are also capable of hydroxylation and epoxidation reactions (Hou et al., 1983; Patel et al., 1983) whereas 1-alkene-utilizing bacteria can only epoxidate double bonds and do not hydroxylate saturated hydrocarbons (de Bont et al., 1979; van Ginkel & de Bont, 1986). Considering the capacity of trans-2-butene-grown Nocardia TB1 to oxidize saturated hydrocarbons, it would seem likely that these cells contain an alkane-type mono-oxygenase with broad substrate specifity, and not an 1-alkene-type mono-oxygenase. Further evidence for an alkane-type mono-oxygenase in trans-2-butene-grown cells was the decrease in oxidation capacity of washed cells during 1-alkene oxidation which was probably caused by the concomittant 1,2-epoxyalkane formation. In Mycobacterium E20, the alkane-mono-oxygenase lost 50% of the original activity at an epoxide concentration of 0.1 mM whereas the alkene mono-oxygenase lost 50% activity at an epoxide concentration which was thirty times higher (Habets-Crützen & de Bont, 1985). Oxidation rates of 1-alkenes in both trans-2-butene- and butane-grown cells were inhibited totally by 0.25 mM epoxide indicating that in trans-2-butene-grown cells also an alkane-type mono-oxygenase is present.

It was possible to detect mono-oxygenase activity in extracts of trans-2-butene-grown Nocardia TB1 and the activity could theoretically result in the formation of either crotonic alcohol or 2-butanone as a result of hydroxylation activity, or in the formation of 2,3-epoxybutane as a result of epoxidation activity of the mono-oxygenase. A hybrid of epoxidation and

hydroxylation of trans-2-butene could also be envisaged, similar to the results of Hou et al. (1983), where oxidation of propene by propane-grown Brevibacterium sp. strain CRL 56 resulted in 1,2-epoxypropane and trace amounts of 3-hydroxy-1,2-propene formation. In the studies of Hou et al. (1983) with extracts of propane-grown Brevibacterium sp. strain CRL 56 it was also shown that 2,3-epoxybutane was formed with trans-2-butene as a substrate for the mono-oxygenase.

1,2-Epoxyethane and 1,2-epoxypropane were not oxidized by trans-2-butene-grown cells whereas 1,2-epoxybutane and especially 2,3-epoxybutane were oxidized which might indicate that trans-2-butene is metabolized via 2,3-epoxybutane. On the other hand, substrate specificity and the inhibition by 1,2-epoxyalkanes formed from 1-alkenes on the 1-alkene oxidation rates indicate that trans-2-butene is metabolized via a hydroxylation reaction. Moreover, results with washed cell suspensions of trans-2-butene-grown Nocardia TB1 showed that 2,3-epoxybutane is not oxidized to CO_2 but to an unidentified product whereas the other possible intermediate crotonic alcohol and trans-2-butene itself were both oxidized to CO_2 (Fig. 3). Indeed, it was shown that washed cells of Nocardia TB1 grown on trans-2-butene excreted a product during the oxidation of 2,3-epoxybutane but this product, which probably originates from a hydroxylation reaction by the mono-oxygenase, was not present in the mineral salts medium of cells growing on trans-2-butene as determined by TOC measurements.

Washed cell suspensions of trans-2-butene-grown cells inhibited with fluoroacetate or arsenite excreted crotonic acid which is an oxidation product of crotonic alcohol, and crotonic alcohol dehydrogenase and crotonic aldehyde dehydrogenase were induced in trans-2-butene-grown cells (Table 3). From these results we conclude that trans-2-butene in Nocardia TB1 is metabolized via an initial hydroxylation at the C_1 position rather than via an initial epoxidation reaction.

It was also possible to detect mono-oxygenase activity in butane-grown cells (Table 3). Hydroxylation of butane could result in either 1-butanol or 2-butanol. Isocitrate lyase activities were present in butane- and 1-butanol-grown cells of Nocardia TB1 whereas in 2-butanol-grown cells isocitrate lyase was absent (Table 4). These isocitrate lyase activities suggest that butane is hydroxylated terminally. Further evidence for the hydroxylation of butane at the C_1 position was obtained using inhibition studies with arsenite. Butyric acid was excreted by butane-grown cells of Nocardia TB1 during the oxidation of butane in the presence of an inhibitor (Fig 2). In cell-free extracts of butane-grown cells of Nocardia TB1, butanal reductase is measured in butane-grown cells of Nocardia TB1

(Table 3).

The further metabolism of trans-2-butene and butane proceeds via β-oxidation as determined by thiokinase and thiolase activities (Table 3).

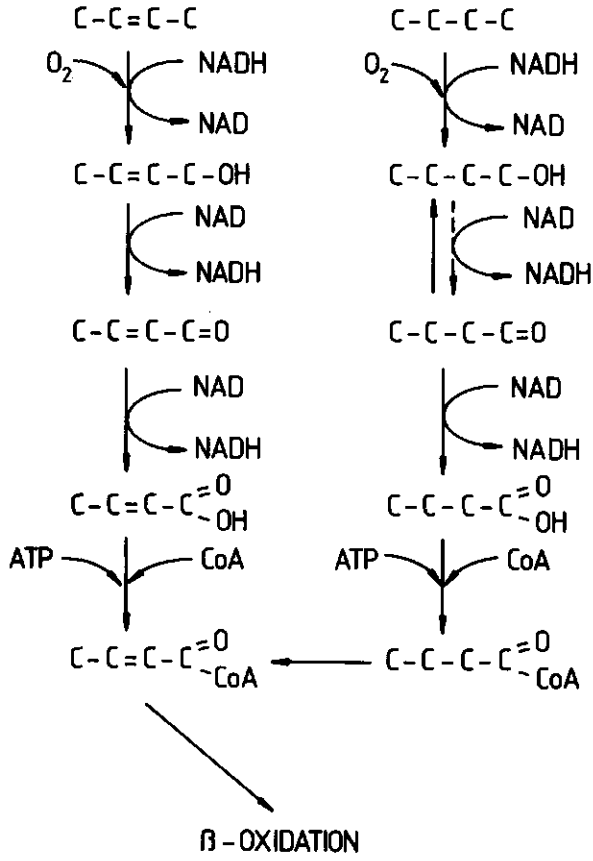

Figure 4. Proposed degradation pathways of trans-2-butene and butane in Nocardia TB1.

Growth of Nocardia TB1 on cis-2-butene was very slow as compared to growth on trans-2-butene although both isomers of 2-butene were oxidized by trans-2-butene- and butane-grown cells at comparable rates (Table 1 and 2). The metabolism of the cis-

isomer was not further investigated also because isomers of crotonic alcohol, crotonic aldehyde and crotonic acid were not available. Literature, to our knowledge, contains no information about the substrate specificity towards the isomers of these compounds of the respective dehydrogenases and thiolase. It is known, however, that crotonyl CoA hydratase which has been isolated in crystalline form catalyses the reversible hydratation of cis- and trans-crotonyl CoA to the corresponding L and D hydroxy butyric acids (Stern & del Campillo, 1956). The next enzyme in the degradative pathway dehydrogenates only the D isomer (Shuster & Doudoroff, 1962; Delafield et al., 1965).

On the basis of the results obtained in this study we propose pathways for the metabolisms of butane and trans-2-butene in Nocardia TB1 as shown in Fig. 4. From our experiments it seems very likely that trans-2-butene degradation is catalysed by the same enzymes as butane degradation because substrate specificities (Table 1 and 2) are comparable and enzymes induced in butane- and trans-2-butene-grown cells are able to oxidize the intermediates of both pathways.

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CHAPTER 5

A DESCRIPTION OF MICROBIAL GROWTH ON GASEOUS ALKENES IN A CHEMOSTAT CULTURE

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SUMMARY

Microbial growth on either ethene or propene was studied in carbon- and energy-limited cultures in a chemostat equipped with a data acquisition and control system. Experimentally determined carbon balances indicated that the cultivation system employed allows accurate measurements. Growth yields and maintenance coefficients for Xanthobacter Py2 on propene and Mycobacterium E3 on ethene were 0.74 Ceq/Ceq and 0.021 Ceq/Ceq.h and 0.50 Ceq/Ceq and 0.011 Ceq/Ceq.h, respectively. The relationship between biomass concentration, alkene concentration in the outlet gas, and dilution rate as well as the productivity of cultures at different dilution rates are described by a model. When experimentally determined growth parameters and calculated K_lA values were used in this model, a good fit for actual biomass productivities was achieved.

Submitted for publication

INTRODUCTION

Alkene-utilizing bacteria have been employed for producing epoxides from gaseous alkenes in either gas/solid1 or in multi-phase bioreactors2,3,. Such organisms contain an inducible alkene mono-oxygenase and consequently need to be cultivated on gaseous substrates such as either ethene or propene when they are used for the production of 1,2-epoxyalkanes. Growing bacteria on gaseous substrates presents some special problems and these have been dicussed by Barnes et al.4 who cultivated methane-utilizing bacteria in chemostats. Particular attention must be paid to the cultivation system employed because, in case of a gaseous substrate, accurate measurements are more difficult than with either miscible liquid or solid substrates. Moreover, the kinetics of gas transfer has to be included when modelling growth kinetics on gaseous substrates. Work with chemostat systems for the growth of bacteria on gaseous substrates has been limited to methane-utilizing bacteria.4-9 To estimate growth parameters of methane-utilizing bacteria in a chemostat culture, Nagai et al.9 used an apparatus in which methane was supplied dissolved in the mineral salts medium, but in all other studies, fermentors were used in which methane was supplied with the air7,8. In our work on bacteria that utilize gaseous alkenes, the gas is also supplied in the gas phase and the main purpose of the present study was to verify the reliability of the system used for estimation of growth parameters of alkene-utilizing bacteria and to describe, with a model, the biomass productivity of cultures growing at different dilution rates. For this we have used two alkene-utilizing bacteria, a Xanthobacter sp.10 and a Mycobacterium sp.11 which were grown in chemostat culture on propene and ethene, respectively.

MATERIALS AND METHODS

Chemicals. Ethene and propene were obtained from Hoek Loos, Amsterdam, The Netherlands. All other chemicals were purchased from Janssen Chimica, Beerse, Belgium.

Micro-organisms. The isolation and characterization of the ethene-utilizing Mycobacterium E3 and the propene-utilizing Xanthobacter Py2 used in this study has been described previously10,11.

Cultivation of the micro-organisms. The organisms were grown at various dilution rates in mineral salts medium12 with the omission of yeast extract, in 1 dm^3 working volume fermentors (Applikon, Vlaardingen, The Netherlands). In case of Xanthobacter Py2 the carbon and energy source was propene and for Mycobacterium E3 the carbon and energy source was ethene. These gases were supplied to the culture by passing a mixture of either 2.3

percent propene or 2.0 percent ethene in air at a rate of 60 cm^3 per minute through the headspace of the vessel. The flow rates of alkene and air were maintained constant with mass flow meters/controllers (Brooks type 5850 TR). The gas flow leaving the fermentor was also measured with a mass flowmeter (Brooks type 5850 TR). The alkene concentrations in the gas entering and leaving the vessel were determined by withdrawing samples from Hungate tubes positioned in the inlet and outlet lines, and analyzing these samples on a gas chromatograph (Packard model 430) as described[12]. The CO_2 and O_2 concentrations in the dried gas outlet were determined with an infrared CO_2 analyzer (Beckman model 864) and a paramagnetic O_2 analyzer (Servomex type OA 250), respectively. Dissolved oxygen concentration (DOC) of the culture was continuously monitored with a steam sterilizable oxygen electrode (Biolafitte). The pH value, measured with a steam sterilizable electrode (Ingold) and registrated with a pH meter (Radiometer), was controlled at pH = 7 with a titrator (Radiometer type 11) by the automatic addition of 0.5 M NaOH. Dilution rates were determined by measuring the medium outflow at various time intervals with a balance (Sartorius). The temperature of the vessel was kept constant at 30 ° C by a thermocontrolled, recycling waterbath (Tamson type TC-3), and the impeller speed was kept constant at 700 rpm. Data for outlet CO_2 and O_2, DOC, gasflow (in and out) and pH were registrated by a HP 86 personal computer equipped with a data acquisition/control unit type 3421A.

Steady state analysis. At steady state (after 3 to 5 volume changes, when alkene consumption, DOC and CO_2 were constant) cells were harvested and TOC was determined: Total Organic Carbon (TOC): TOC of the culture biomass and of the culture supernatant was analyzed with a TOC analyzer (Dohrman, type DC).

Carbon balances. The carbon recovery was estimated from the amount of carbon (ethene or propene) consumed by the organism (expressed as mg C per h) in the vessel and the amount of carbon, CO_2 and biomass, (expressed as mg C per h) leaving the vessel.

Determination of the yield on ethene, propene, acetic acid and ethanol in batch culture. Xanthobacter Py2 and Mycobacterium E3 were grown in 0.5 dm^3 mineral medium in a 5 dm^3 Erlenmeyer flask closed with a rubber stopper fitted with a suba seal and 200 cm^3 alkene was injected through the suba seal or 0.3% (w/v) acetic acid or 0.5% (v/v) ethanol was added to the mineral salts medium.

RESULTS AND DISCUSSION

Carbon balances

Mycobacterium E3 and Xanthobacter Py2 were grown in continuous culture under ethene- and propene-limitation respectively, It was verified that cultures were alkene-limited by doubling the concentration of alkene in the gas supply and by recording a two-fold increase in the optical density of the culture.

Carbon balances obtained for a number of dilution rates of ethene-limited Mycobacterium E3 and propene-limited Xanthobacter Py2 cultures are given in Table 1 and 2, respectively. No appreciable amounts of organic carbon were detected in the

Table 1. Percentages of carbon recovery for an ethene-limited culture of Mycobacterium E3 and C-balances at various dilution rates.

D	C_2H_4cons	CO_2 prod	TOCbiomass[1]	Crec
(h-1)	mg C h-1	mg C h-1	mg C h-1	%
0.014	27.5	15.1	10.9	95
0.025	29.5	15.7	11.3	92
0.032	29.5	15.7	12.3	95
0.036	29.5	15.7	13.6	99

1) TOCsupernatant values were always less than 0.3 mg C h^{-1}.

Table 2. Percentages of carbon recovery for a propene-limited culture of Xanthobacter Py2 and C-balances at various dilution rates.

D	C_3H_6cons	CO_2 prod	TOCbiomass[1]	Crec
(h-1)	mg C h-1	mg C h-1	mg C h-1	%
0.035	48.5	23.1	26.3	102
0.039	47.3	22.3	25.9	102
0.059	48.8	26.2	22.3	98
0.095	48.0	27.1	18.1	94
0.101	48.0	27.3	17.2	92
0.134	47.4	28.0	18.1	98

1) TOCsupernatant values were always less than 0.3 mg C h^{-1}.

supernatant fluids so it may be concluded that the alkenes metabolized by the organisms were totally used for production of cells and CO_2. The Crec% is typical and the results were considered as reliable since the Crec%'s were all between 90% and 105%. These results imply that the fermentation system used throughout this investigation allows measurements of both gas-flows and of concentrations of gases in the gas stream with a sufficient degree of accuracy.

Growth-parameters: determination of Y_{max}, m_s, K_s and μ_{max}.

As indicated by Fieschko and Humphrey[13], the true growth yields (Y_{max}) and maintenance coëfficients (m_s) can be most accurately determined from the following equations[14].

$$q = D/Y_{max} + m_s \qquad (1)$$

and

$$1/Y_s = 1/Ymax + m_s/D \qquad (2)$$

The Y_{max} for Xanthobacter Py2 growing on propene and for Mycobacterium E3 growing on ethene, calculated from equation 1, are 0.50 Ceq/Ceq and 0.74 Ceq/Ceq, respectively. These Y_{max} values obtained in continuous culture are higher than the Y_s values obtained in batch culture i.e., Y_s = 0.40 Ceq/Ceq for Mycobacterium E3 and Y_s = 0.59 Ceq/Ceq for Xanthobacter Py2. Values obtained for Mycobacterium E3 and Xanthobacter Py2 are compared in Table 3 with results from the literature. The true growth yield for ethene is approximately equal to reported Y_{max} values of bacteria growing on acetic acid and is lower than the reported Y_{max} values for bacteria growing on ethanol and methanol (Table 3). Ethene has the same degree of reduction as ethanol and methanol, implying that the Y_{max} value for growth on ethene should be about the same as the Y_{max} value for growth on ethanol and methanol. However, the first step in the pathway for ethene metabolism is a NADH-consuming enzyme reaction[15] whereas the metabolism of the alcohols is only energy-generating. Therefore, the Y_{max} value on ethene will be comparable to the Y_{max} value on acetate. Similar arguments apply when comparing Y_{max} values on methane to Y_{max} values on methanol. Methane is more reduced than methanol, but the first step in methane metabolism is also energy-demanding. As a consequence Y_{max} on methanol will be higher than Y_{max} on methane (Table 3). It is not possible to compare Y_{max} values of Xanthobacter Py2 on propene with other Y_{max} values because the metabolic pathway of propene is only partly known[1].

The values for m_s for growth of Mycobacterium E3 on ethene and for growth of Xanthobacter Py2 on propene calculated from equation 2, 0.011 Ceq ethene per Ceq biomass per h and 0.021 Ceq propene per Ceq biomass per h, respectively, are comparable with reported values for growth on methane, ethanol and acetic acid, but maintenance coefficients for methanol in general are higher (Table 3).

The generally used expression for relating substrate concentration in the liquid phase to bacterial growth rate in chemostat cultures is[16]:

$$C_l = K_s.D \ / \ (\mu max - D) \qquad (3)$$

Equation (3) can be used to establish a model which, at a fixed alkene concentration in the inflowing gas, relates biomass concentration to both the concentration of alkene in the outflowing gas and the dilution rate. Unfortunately, it was impossible to experimentally determine the saturation constant (K_s) in chemostat culture because of the very low value of K_s

Table 3. Reported growth yields and maintenance coefficients for several micro-organisms grown in continuous cultures or batch cultures on carbon and energy sources with various degrees of reduction.

Organism	Substrate	Y_{max}	m_s	R_s	Ref.
Methylomonas methanooxidans	methane	0.721	-	8	24
Methylococcus capsulatus	methane	0.651	-	8	24
Pseudomonas sp.	methane	0.661	-	8	25
Methylococcus capsulatus	methane	0.651	-	8	8
Methylococcus sp.	methane	0.56	0.03	8	25
Methane bacterium	methane	0.65	0.03	8	-
Pseudomonas C	methanol	0.80	0.061	6	26
Pseudomonas methylotropha	methanol	0.77	0.096	6	26
Methylomonas methanolica	methanol	0.63	0.15	6	27
Pseudomonas fluorescens	ethanol	0.461	-	6	28
Pseudomonas fluorescens	acetate	0.341	-	4	28
Pseudomonas sp.	acetate	0.471	-	4	29
Mycobacterium E3	ethene	0.50	0.011	6	-
Mycobacterium E3	ethene	0.401	-	6	-
Mycobacterium E3	acetate	0.441	-	4	
Mycobacterium E3	ethanol	0.531	-	6	
Xanthobacter Py2	propene	0.74	0.025	6	-
Xanthobacter Py2	propene	0.591	-	6	-

Growth yields determined in batch culture.

and because of the immediate transfer of the gaseous alkenes from the gas phase to the aqueous phase when a sample was drawn from the chemostat. However, reliable values for the Michaelis Menten constants (K_m) for ethene of Mycobacterium E3 and propene of Xanthobacter Py2 have been determined in dilute resting-cell suspensions by following the disappearance of the alkenes from the gas phases. The K_m value for ethene of Myco-

bacterium E3 was 100 vpm[17] and the K_m value for propene of Xanthobacter Py2 was 280 vpm[10]. K_m values for the liquid phase can be calculated from the solubility values for both ethene and propene at 30°C in water at 100 percent saturation (3.9 mol/m^3 and 4.6 mol/m^3, respectively)[18,19]. These, K_m values are 3.9 10^{-7} mol/dm^3 for ethene and 1.3 10^{-6} mol/dm^3 for propene. Although these values are valid for resting-cell suspensions, it is a reasonable assumption that values for K_s constants of growing cells are similar since it is probable that the first step in the alkene-pathway is growth rate-limiting. K_s values for ethene and propene are lower than values observed for other carbon and energy sources, including methane[20]. Reported values of the K_s for methane are 2.6 10^{-5} mol/m^3[21] and 1.9 10^{-5} mol/m^3[22].

The maximum growth rate of both the ethene- and propene-utilizing strains was determined in batch experiments. These maximum growth rates were 0.09 h^{-1} and 0.15 h^{-1}, respectively.

Model of microbial growth on gaseous substrates.

To develop and evaluate a simple model describing the growth of Xanthobacter Py2 in a chemostat on propene, the following measured parameters and a calculated K_lA were used: K_s = 6 10^{-3} Ceq/m^3; f = 6.3 10^{-3} m^3/h; V = 1.9 m^3; Y_{max} = 0.74 Ceq/Ceq ; M_s = 0.021 Ceq/Ceq h ; K_lA = 8.8 h^{-1} ; H = 8.6 ; C_{gi} = 2.8 Ceq/m^3 ; μ_{max} = 0.15 h^{-1}. As stated the only values not measured were the K_s which was predicted from K_m values for washed cells. The K_lA was calculated from the measured alkene concentrations in the in- and outflowing gas and by assuming the alkene concentration in the water phase was zero.

In our chemostat experiments the carbon and energy source is a gas which is not supplied with the liquid medium but with the airflow. This implies that the amount of substrate available is not proportional to the dilution rate. Therefore, the biomass-dilution rate plot differs from the plot obtained with usual chemostat studies in which the carbon and energy source is supplied in the medium and the relationship between the biomass concentration and the dilution rate resembles that resulting from equation (4) proposed by Harrison[23] for oxygen limited cultures, i.e.

$$X = K_lA \cdot (C_s - C_l) \cdot Y_s / D \qquad (4)$$

From a second mass balance equation for the fermentor the biomass also can be calculated with equation (5)

$$X = (C_{gi} - C_{go}) \cdot Y_s \cdot f / D \cdot V \qquad (5)$$

In equation (5) it is assumed that the flows of in- and outflowing gas are equal and this assumption is valid because the measured flows did not differ by more than 5 percent from each other. From equation (4) and (5) and Henry's law, it follows that the concentration of the alkene in the outflowing gas at various dilution rates is given by equation (6):

$$C_s = C_{go}.H = (C_{gi}.f/V) + (K_lA.C_l) / (K_lA+f/V.H) \quad (6)$$

When the K_s of the alkene-utilizing bacterium is very low, it may be expected that at a fixed C_{gi}, the alkene concentration in the outflowing gas, C_{go}, will be the same at all dilution

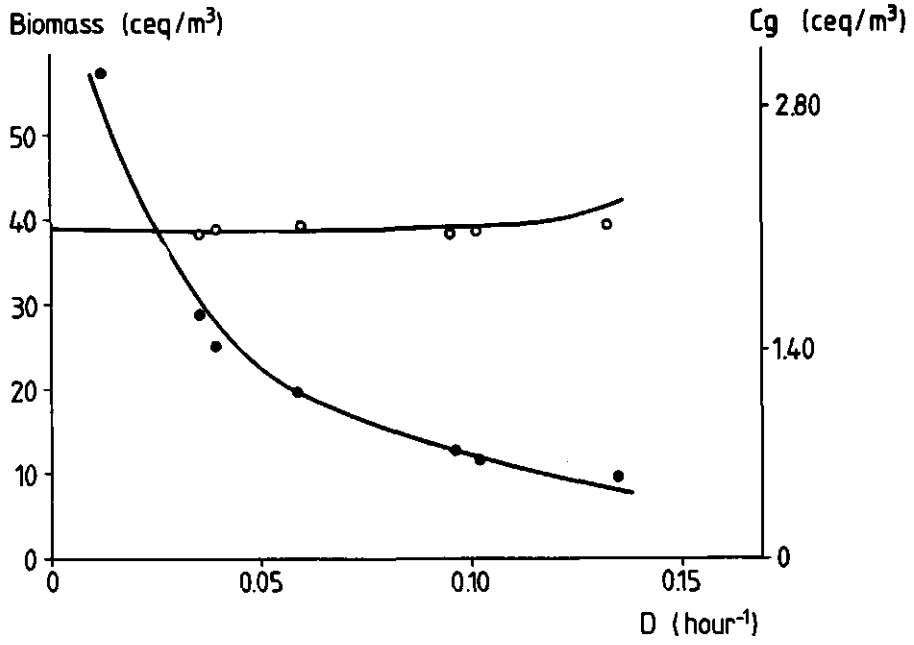

Figure 1. Measured and calculated relationships between the biomass (●) and the outflowing propene concentration (○) and the dilution rate during growth on 2.3 percent propene in the gas phase. The solid lines represent the calculated biomass concentration and the calculated propene concentration in the outflowing gas from the following parameters : f = 6.3 10^{-3} m^3/h; V = 1.9 10^{-3} m^3; Y_{max} = 0.74 Ceq/Ceq; M_S = 0.021 Ceq/Ceq.h; μ_{max} = 0.15 h^{-1}; K_lA = 8.8 h^{-1}; C_{gi} = 2.8 Ceq/m^3 ; K_s = 12 10^{-3} Ceq/m^3 ; H = 8.6 m^3(gas)/m^3(liquid).

rates except these close to wash out. Experimental results with

Mycobacterium E3 and Xanthobacter Py2 verify this relation between the alkene concentration in the outflowing gas and the dilution rate as can be seen from Fig. 1. The assumption of a high affinity of the bacteria for alkenes by assuming $K_s = K_m$ is probably valid.
From equation (1) and equation (4) the biomass concentration at different dilution rates can be calculated:

$$X = Y_{max} \cdot K_1A \cdot (C_s - C_1) / (Y_{max} \cdot (m + D)) \qquad (7)$$

This relationship differs from that which applies for conventional chemostats, in that the biomass concentration is highest at low growth rates and falls off steeply as the growth rate increases. The steady state values obtained for biomass concentration at different dilution rates are in agreement with these calculated values for the biomass concentrations as shown in Fig. 1.

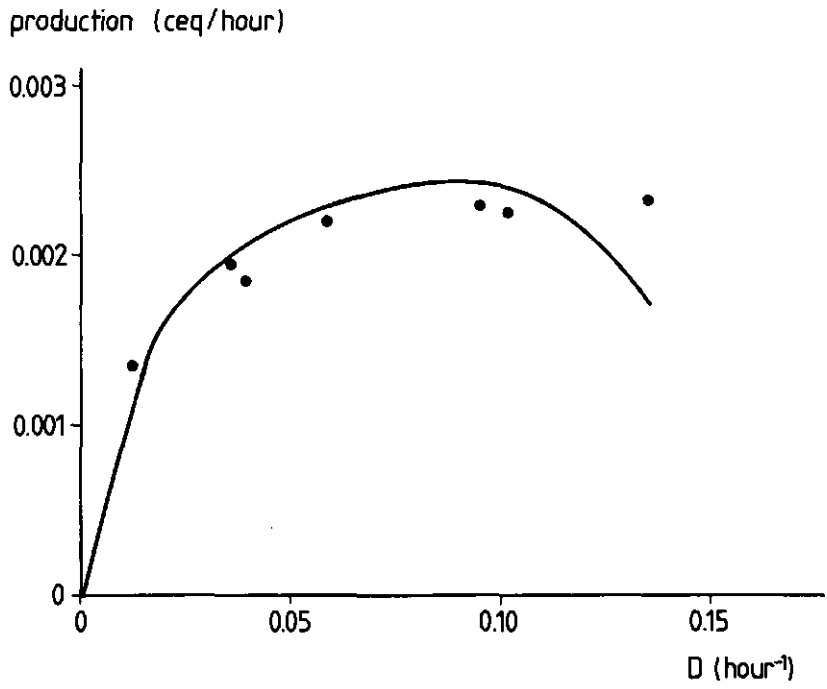

Figure 2. Relationship between the productivity (●) of Xanthobacter Py2 on propene and different dilution rates. The solid line represents the calculated production rate from the values given in Fig. 1.

In a conventional chemostat, the optimum dilution rate for the productivity of biomass is near wash-out conditions. In a chemostat with gaseous substrates, productivity was found to be maximal and nearly constant at dilution rates varying from 0.5 μmax to near μmax as can be seen from Fig. 2. Our productivity is about half the published values for productivities in chemostats with methane as substrate[7,8], and this is probably due to poor mass transfer of the gaseous substrates in the fermentors used.

NOMENCLATURE

Cl	alkene concentration in the liquid phase (mol/m^3)
Cs	alkene concentration in the liquid phase saturated with the outcoming gas (mol/m^3)
Cgi	alkene concentration of the incoming gas (mol/m^3)
Cgo	alkene concentration of the outcoming gas (mol/m^3)
Ceq	carbon equivalents
Ks	Monod constant (Ceq/m^3)
μmax	maximum growth rate (h^{-1})
D	dilution rate (h^{-1})
Ys	growth yield (Ceq/Ceq)
Ymax	maximum true growth yield (Ceq/Ceq)
ms	maintenance coefficient (Ceq/Ceq.h)
q	metabolic quotient (Ceq/Ceq.h)
KlA	volumetric mass transfer rate (h^{-1})
X	biomass concentration (Ceq/m^3)
f	gas flow rate (m^3/h)
V	working volume of the fermentor (m^3)
Km	concentration of the half maximum of the alkene oxidation rate by resting-cell suspensions (mol/m^3)
Crec	percentage of carbon recovery (%)
H	Henry coefficient m^3 (gas)/ m^3 (liquid)
Rs	generalized degree of reduction of substrate
TOC	total organic carbon
DOC	dissolved oxygen concentration

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CHAPTER 6

EPOXIDATION OF ALKENES BY ALKENE-GROWN *XANTHOBACTER* spp.

C.G. van Ginkel, H.G.J. Welten and J.A.M. de Bont

SUMMARY

Newly isolated *Xanthobacter* spp. were able to grow on the gaseous alkenes like ethene, propene, 1-butene and 1,3-butadiene. Resting-cell suspensions of propene-, 1-butene- or 1,3-butadiene-grown *Xanthobacter* Py10 accumulated 1,2-epoxyethane from ethene. Ethene-grown *Xanthobacter* Py10 did not produce any 1,2-epoxyalkane from the alkenes tested. Furthermore, propene-grown *Xanthobacter* Py2 accumulated 2,3-epoxybutane from *trans*-2-butene and *cis*-2-butene but did not form epoxides from other substrates tested.

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INTRODUCTION

The formation of 1,2-epoxyalkanes from alkenes has been studied in many micro-organisms. Most of these micro-organisms able to epoxidate alkenes are alkane-utilizing bacteria. The epoxidation of an alkene was first demonstrated by van der Linden (1963) who detected the formation of 1,2-epoxyoctane from octene by heptane-grown Pseudomonas cells. The epoxidation of alkenes by alkane-utilizing Pseudomonas cells was also reported by several other authors (Abbott and Hou 1973; de Smet et al. 1981; 1983) and an octane-utilizing Corynebacterium also excreted epoxides in this way (Cardini and Jurtshuk 1970).

However, none of these bacteria were able to epoxidate gaseous alkenes. Hou et al. (1983) and Patel et al. (1983) reported that ethane-, propane- and butane-metabolizing bacteria excreted 1,2-epoxyalkanes from gaseous alkenes. Methanotrophic bacteria also catalysed the epoxidation of gaseous alkenes (Hou et al. 1979; Higgins et al. 1979; 1980), and the methane-grown Methylosinus trichosporium was even found to be active on cycloalkanes as well as on aromatic compounds. The epoxidation of alkenes by these alkane-grown bacteria is due to the non specific alkane hydroxylase that is able to oxidize alkanes as well as alkenes.

Several micro-organisms isolated on alkenes have also been tested for their ability to epoxidate alkenes and for this purpose bacteria of the genera Mycobacterium (Habets-Crützen et al. 1984) and Nocardia (Furuhashi et al. 1981) grown on ethene or propene were used. The ethene- and propene-grown mycobacteria were able to accumulate 1,2-epoxypropane and 1,2-epoxyethane, respectively (Habets-Crützen et al. 1984). Formation of epoxides by these bacteria is due to the substrate specificity of the 1,2-epoxyalkane degrading enzymes (Habets-Crützen et al. 1984). Furuhashi et al. (1981) reported that Nocardia corallina B276 accumulated 1,2-epoxypropane during growth on propene. Because the production rate of 1,2-epoxyalkanes by the mycobacteria is rather low, we have tried to isolate some faster growing alkene-utilizing micro-organisms. Some fast growing Xanthobacter spp. isolated on propene or 1-butene was the result of this attempt (van Ginkel and de Bont 1986).

These organisms are especially interesting as potential epoxide producers because some of the epoxides were formed in high enantiomeric excess (Habets-Crützen et al. 1985). In this paper we present more detailed results on the accumulation of 1,2-epoxyalkanes from alkenes by ethene-, propene-, 1-butene- and 1,3-butadiene-grown Xanthobacter.

MATERIALS AND METHODS

Chemicals. Gaseous alkenes and 1,2-epoxyethane were obtained from Hoek Loos, Amsterdam, The Netherlands. All other chemicals were obtained from Janssen Chimica, Beerse, Belgium.

Cultivation of the micro-organism. The micro-organisms were cultivated in mineral salts medium supplemented with the appropriate gaseous alkenes as described by Wiegant and de Bont (1980). Growth on different carbon sources was determined at 30°C in 100 cm^3 Erlenmeyer flasks containing 10 cm^3 mineral medium supplemented with the appropriate carbon source.

Analyses. Determination of gaseous alkenes and 1,2-epoxyalkanes has been described by de Bont et al. (1979). Protein concentration of washed cell suspensions was determined as described by Habets-Crützen et al. (1984).

Determination of the doubling times. The culture doubling times were measured as described by Habets-Crützen et al. (1984).

Oxidation of hydrocarbons and 1,2-epoxyalkanes. Preparation of washed cell suspensions has been described by de Bont and Harder (1978). The oxidation of hydrocarbons and the excretion of 1,2-epoxyalkanes by these washed cell suspensions were measured by incubating the cells in a 50 mM phosphate buffer (pH = 7.0) at 30°C in Hungate tubes. The appropriate gas (0.5 cm^3) or 0.1 cm^3 of a 100 mM 1,2-epoxyalkane solution in a 50 mM phosphate buffer (pH = 7.0) was injected in the Hungate tubes. Samples from the gas phase were withdrawn at regular intervals and analysed for hydrocarbons and 1,2-epoxyalkanes.

RESULTS

Growth characteristics on hydrocarbons

The alkene-utilizing Xanthobacter spp. studied have been isolated on either propene (Py2, Py3, Py7, Py10, Py11, Py17) or 1-butene (By2) (van Ginkel and de Bont 1980) and they were tested for growth on several other hydrocarbons. They did not grow on saturated hydrocarbons like methane, ethane, propane, butane, hexane, cyclohexane and hexadecane. Growth on unsaturated hydrocarbons like trans-2-butene, cis-2-butene, allene, ethyne and propyne did not occur either. Only ethene, propene, 1-butene and 1,3-butadiene supported growth. The culture doubling times of these organisms on propene and 1-butene ranged from 5 to 7 h, but growth on ethene and 1,3-butadiene was much slower. Xanthobacter Py10 was the fastest growing strain on ethene and 1,3-butadiene, and culture doubling times of this organism on ethene and 1,3-butadiene were 25 and 32 h, respectively.

Substrate specificity and excretion of 1,2-epoxyalkanes

Resting-cell suspensions of propene-grown Xanthobacter were

able to oxidize ethene, propene and 1-butene at rates varying

Table 1. Oxidation of alkenes and accumulation of 1,2-epoxyethane by washed cell suspensions of propene-grown Xanthobacter strains. None of the strains excreted 1,2-epoxypropane or 1,2-epoxybutane from propene or 1-butene.

Strain	Substrate oxidation rate1			Product accumulation rate1
	Ethene	Propene	1-Butene	1,2-Epoxyethane
Py2	55	83	67	46
Py3	53	81	77	27
Py7	35	65	47	29
Py10	40	66	58	29
Py11	43	75	60	21
Py17	38	77	54	25
By2	43	79	46	20

1) in nmol per minute per mg protein

Table 2. Oxidation of alkenes and 1,2-epoxyalkanes and the accumulation of 1,2-epoxyalkanes by ethene-, propene-, 1-butene- and 1,3-butadiene-grown Xanthobacter Py10 cells.

Growth substrate	Ethene	Propene	1-Butene	1,3-Butadiene
Oxidation rate1				
Ethene	20	40	39	11
Propene	30	66	65	20
1-Butene	29	64	63	21
1,3-Butadiene	11	17	16	9
1,2-Epoxyethane	15	10	13	3
1,2-Epoxypropane	25	60	58	20
1,2-Epoxybutane	25	62	60	18
3,4-Epoxy-1-butene	10	18	15	10
Accumulation rate1				
1,2-Epoxyethane	0	29	24	4
1,2-Epoxypropane	0	0	0	0
1,2-Epoxybutane	0	0	0	0
3,4-Epoxy-1-butene	0	0	0	0

1) in nmol per minute per mg protein.

from 38 to 83 nmoles of alkene oxidized per minute per mg protein (Table 1). The propene-grown Xanthobacter spp. oxidized propene and 1-butene without a transient extracellular production of the corresponding 1,2-epoxyalkanes but from ethene these whole cell suspensions excreted 1,2-epoxyethane. The production of 1,2-epoxyethane from ethene was not stoichiometric (Table 1). The behaviour of these micro-organisms towards alkenes was investigated in more detail by comparing washed cell suspensions of Xanthobacter Py10 grown on either ethene, propene, 1-butene and 1,3-butadiene. Xanthobacter Py10 was used because this strain grew fastest on ethene and 1,3-butadiene. Oxidation rates for ethene, propene, 1-butene and 1,3-butadiene were lower in ethene- and 1,3-butadiene-grown cells than in propene- and 1-butene-grown cells but in all cases the ratios of oxidation rates of ethene, propene, 1-butene and 1,3-butadiene of the ethene- and 1,3-butadiene-grown cells were comparable to the ratios of propene- and 1-butene-grown cells. A different pattern was observed for the oxidation of 1,2-epoxyalkanes by alkene-grown Xanthobacter Py10. 1,2-Epoxyethane was relatively poorly oxidized by cells grown on propene, 1-butene or 1,3-butadiene as compared with ethene-grown cells. The ethene-grown cells oxidized all 1,2-epoxyalkanes at rates that were comparable with the alkene oxidation rates. Consequently propene-, 1-butene- and 1,3-butadiene-grown cells excreted 1,2-epoxyethane but no other 1,2-epoxyalkanes, while ethene-grown cells did not accumulate epoxides at all (Table 2).

Substrate specificity towards other compounds

The substrate specificity towards other hydrocarbons was tested with propene-grown Xanthobacter Py2 cells. Trans-2-butene, cis-2-butene, 1-pentene, 1-hexene, and isoprene were oxidized, but epoxide accumulation was only detected during the oxidation of trans-2-butene or cis-2-butene (Table 3). The 2,3-epoxybutanes were not formed in stoichiometric amounts. Although cis-2-butene was oxidized faster than trans-2-butene, the 2,3-epoxybutane accumulation rate from trans-2-butene was more than twice as high as from cis-2-butene (table 3). Other unsaturated hydrocarbons like allene, ethyne, propyne, limonene and myrcene were not oxidized. Furthermore, propene-grown Xanthobacter Py2 did not oxidize aromatic compounds like benzene, xylene, styrene and toluene or saturated hydrocarbons like methane, ethane, propane, butane, pentane, hexane and cyclohexane. Xanthobacter Py2 did not oxidize CO either.

Table 3. Oxidation of unsaturated hydrocarbons and the formation of epoxyalkanes by washed cell suspensions of propene-grown Xanthobacter Py2.

Substrate	Substrate oxidation rate[1]	Product detected	Product accumulation rate[1]
trans-2-butene	60	2,3-Epoxybutane	41
cis-2-Butene	71	2,3-Epoxybutane	17
1,3-Butadiene	30	None	-
1-Pentene	18	None	-
Isoprene	4	None	-
1-Hexene	2	None	-

1) in nmol per minute per mg protein.

DISCUSSION

Bacteria growing on either ethene, propene, 1-butene or 1,3-butadiene have been described by several authors (de Bont 1976; Heyer 1976; Cerniglia et al. 1976; Watkinson and Sommerville 1976) but until recently only bacteria belonging to the genera Mycobacterium (de Bont et al. 1980) and Nocardia (Watkinson and Sommerville 1976) had been isolated on these gaseous alkenes. Recent Xanthobacter isolates grew on both ethene, propene, 1-butene and 1,3-butadiene while the other alkene-utilizing micro-organisms grew only on a limited number of these gaseous alkenes (Habets-Crützen et al. 1984). A few alkene-utilizing Mycobacterium (de Bont et al. 1979) and Nocardia (Fujii et al. 1985) isolates were able to metabolize gaseous alkanes as well, but the alkene-utilizing Xanthobacter were not able to grow on saturated hydrocarbons, although other Xanthobacter have been isolated on cyclohexane (Trower et al. 1985) and butane (Coty 1967).

The alkene oxidation rates of Xanthobacter were higher than the oxidation rates of alkene-grown Mycobacterium (Habets-Crützen et al. 1984) which is probably a reflection of the higher growth rate of the Xanthobacter. These oxidation rates of the Xanthobacter are of the same order of magnitude as the activities of methane-, ethane-, propane- and butane-utilizing bacteria (Hou et al. 1979; Stirling and Dalton 1979; Hou et al. 1983; Patel et al. 1983). Oxidation rates of ethene, propene, 1-butene and 1,3-butadiene by cells of Xanthobacter Py10 grown on these four alkenes suggest that these alkenes were oxidized by the same enzyme. However, the further metabolism of epoxides in Xanthobacter Py10 is probably mediated by two different 1,2-epoxyalkane degrading enzymes because ethene-grown cells

oxidized all 1,2-epoxyalkanes at the same rate whereas cells grown on propene or 1-butene oxidized 1,2-epoxypropane and 1,2-epoxybutane six times faster than 1,2-epoxyethane. Consequently, the Xanthobacter strains excreted 1,2-epoxyethane from ethene although not in stoichiometric amounts as did propene-grown Mycobacteria (de Bont et al. 1983; Habets Crützen et al. 1984). Unfortunately , neither 1,2-epoxypropane nor 1,2-epoxybutane accumulated from the respective alkenes. In Xanthobacter Py2 the oxidation of other unsaturated hydrocarbons was also tested and only from trans-2-butene and cis-2-butene an epoxide was detected. In both cases the formation of 2,3-epoxybutanes again was not in stoichiometric amounts. Higher 1-alkenes were also oxidized but at considerable lower rates. The substrate specificity of propene-grown Xanthobacter Py2 towards hydrocarbons is different from the substrate specificity of alkane-utilizing bacteria but resembles the substrate specificity of Mycobacteria. Alkane-metabolizing micro-organisms are able to hydroxylate alkanes and epoxidate alkenes while alkene-utilizing Xanthobacter and Mycobacteria are only capable of epoxidating alkenes (Habets-Crützen et al. 1984). CO is not oxidized by propene-grown Xanthobacter Py2 whereas methanotrophic bacteria are able to oxidize this compound (Higgins et al. 1979).

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CHAPTER 7

OXIDATION OF GASEOUS AND VOLATILE HYDROCARBONS BY SELECTED ALKENE-UTILIZING BACTERIA

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SUMMARY

Most gaseous and volatile alkenes are susceptible to microbial degradation. Especially bacteria belonging to the genera Mycobacterium, Nocardia and Xanthobacter play a role in this degradation. Eleven strains of these alkene-utilizing bacteria were tested for their ability to grow in a mineral salts medium with C_1-C_6 alkanes, C_2-C_6 alkenes, alkadienes and monoterpenes furnished individually as sole sources of carbon and energy. A limited number of alkenes and alkanes supported growth of the bacteria; some bacteria were unable to grow on any of the saturated hydrocarbons tested. Surprisingly, monoterpenes were frequently used as carbon and energy sources by alkene-utilizing bacteria belonging to the genera Mycobacterium and Nocardia. Washed cell suspensions of alkene-grown bacteria were able to attack the whole range of alkenes tested, whereas only three strains were able to oxidize alkanes as well. Alkenes tested were oxidized either to water and carbon dioxide or to epoxyalkanes. Few epoxides accumulated in stoichiometric amounts from the corresponding alkenes, because most epoxides formed were further converted to other compounds e.g. alkanediols.

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INTRODUCTION

In nature, several gaseous and volatile alkenes are produced. The most predominant of these compounds are the gaseous plant hormone ethene (2,30), the foliage volatile isoprene (31) and various monoterpenes that are present in plant oils. Many unsaturated hydrocarbons, especially the lower gaseous alkenes ethene, propene, 1,3-butadiene and butenes are produced chemically on a large scale and inevitably these compounds are partly released into the environment. It is therefore not surprising that many micro-organisms have been isolated that are able to use these compounds as carbon and energy sources. Isolation substrates included ethene (3,19), propene (5,8,13), 1,3-butadiene (15,36), 2-butene (14), and monoterpenes as for instance myrcene (28) and α-pinene (24,39). Other micro-organisms, which were isolated using substrates such as alkanes, have also been tested for their ability to grow on alkenes but growth on unsaturated hydrocarbons was recorded in a very limited number of instances only (9,10,26).

Nevertheless, resting cells of alkane-grown bacteria, including methane-utilizers, often were able to epoxidate alkenes due to the broad substrate specificity of alkane mono-oxygenase which is responsible for the initial oxidation of alkanes (20, 21,23,29,33). Alkene-utilizers also contain mono-oxygenases with a broad substrate specificity but enzymes from these organisms generally do not hydroxylate alkanes (4,7,12).

Alkene oxidation by washed cells of either alkane- or alkene-grown cells very often resulted in the formation and excretion of epoxides. Examples of epoxide-forming alkane-utilizers are resting cells of methane- and alkane-grown bacteria that form these compounds from alkenes as a consequence of either the inability of these bacteria to degrade epoxides (22) or of a negligible oxidation rate of epoxyalkanes (29). The excretion of epoxides by alkene-utilizing bacteria is a consequense of a restrictive range of substrates utilized by the epoxyalkane-degrading enzymes (16). Accumulation of epoxides during growth of an organism was shown by Furuhashi et al. (11) who detected 1,2-epoxypropane accumulation during growth of Nocardia corallina B-276 on propene.

Epoxyalkane-producing micro-organisms have frequently been considered as potential biocatalysts in biotechnological processes for the production of epoxides. Alkene-utilizing bacteria should then be preferred over alkane-utilizing organisms for several reasons. (i) Alkene-utilizing bacteria form epoxides in high enantiomeric excess (18), whereas methane-grown bacteria produce racemic epoxyalkanes (34). (ii) Alkene-grown

bacteria only epoxidate and do not hydroxylate alkenes as do some alkane-utilizers and (iii) alkane-grown bacteria are more sensitive to epoxyalkanes than alkene-utilizing bacteria (17).

In view of the role alkene-utilizing organisms play in nature, and in view of their potential application in epoxide production, it seems desirable to obtain a more comprehensive knowledge of these bacteria. We therefore compared several available alkene-utilizing bacteria (5,13,14,15,16) and two new isolates. This paper particularly deals with the capacity of these selected organisms to form and excrete epoxyalkanes from alkenes.

MATERIALS AND METHODS

Chemicals. All gaseous alkenes and 1,2-epoxyethane were obtained from Hoek Loos, Amsterdam, The Netherlands. All other chemicals were purchased from Janssen Chimica, Beerse, Belgium.

Micro-organisms. The isolation and description of the bacteria used was reported earlier (5,13,14,15,16). Nocardia H8 and Pseudomonas H1 were isolated by similar methods except that 1-hexene was used as sole source of carbon and energy (3).

Cultivation of the micro-organism. Organisms were grown at 30°C in 5 dm^3 Erlenmeyer flasks containing 500 cm^3 mineral salt medium (37) with the gaseous alkene in air (5%) or 1 cm^3 of a volatile alkene as the sole carbon and energy source.

Analyses. Determination of alkenes, 1,2-epoxyalkanes, 1,2-propanediol and carbon dioxide was described previously (4,7,37). Protein concentration of washed cell suspensions was determined as described by Habets-Crützen et al. (16). Mycolic acids were determined by thin layer chromatographic analysis of whole organisms methanolysates as described by Minnikin et al. (27).

Growth of micro-organisms. Micro-organisms were grown on slopes of mineral salts medium described previously (37). These slopes were placed in a dessicator and the appropriate gas was injected or a volatile alkene in a test tube was placed in the dessicator. After three weeks the slopes were examined for growth.

Oxidation of hydrocarbons. Preparation of washed cell suspensions has been described by de Bont et al. (4). 2 Cm^3 of cell suspension was placed in 30 cm^3 screw-cap bottles. The appropriate gas (0.5 cm^3) or volatile compound (0.3 10^{-3} cm^3) was injected in the screw-cap bottles. The reaction mixture was incubated at 30°C on a water-bath rotary shaker at 150 rpm and alkenes and the products of epoxidation were assayed at regular intervals.

Conversion of 1,2-epoxypropane to 1,2-propanediol. A washed cell suspension of ethene-grown Mycobacterium E3 (10 cm^3) was incubated with 0.25 mM 1,2-epoxypropane. During 1,2-epoxypropane degradation by washed cell suspensions of ethene-grown Mycobacterium E3, CO_2 formation was measured

along with the endogenous CO_2 formation rate. At regular intervals the epoxide concentration was determined by analyzing the headspace gas chromatographically. After centrifugation, samples of washed cell suspensions were analysed for 1,2-propanediol.

RESULTS

Micro-organisms growing on gaseous and volatile alkenes

Previously, several micro-organisms including Mycobacterium, Nocardia and Xanthobacter strains have been isolated on gaseous and volatile alkenes. Due to the natural occurrence of ethene, this compound was used most extensively as a carbon and energy source to isolate alkene-utilizing bacteria (3,16,19). Other alkenes used were propene, 1-butene and 1,3-butadiene (8,13, 36). More recently, trans-2-butene- and isoprene-utilizing bacteria have been isolated (14,15).

We now have isolated several other alkene-utilizing bacteria using soil samples from ten different locations. The enrichment cultures were set up individually with alkenes used previously (3,13,15,16) or with other alkenes like allene, 1-pentene and 1-hexene. In general, all enrichment cultures showed growth within a week except for incubations with allene, trans-2-butene and 1-pentene. In spite of numerous efforts, a 1-pentene-utilizing bacterium was not isolated and using allene as carbon and energy source, it was not even possible to obtain a positive enrichment culture. Out of ten enrichment cultures, only three trans-2-butene-utilizing bacteria were isolated. Many Pseudomonas spp. and Nocardia H8 were enriched and subsequently isolated with 1-hexene as carbon and energy source. All newly isolated bacteria were tentatively classified on basis of Gram-staining, microscopic observation and mycolic acid analysis. Table 1 summarizes the genera to which the new isolates were assigned and gives the number of strains isolated.

A representative selection of 11 alkene-utilizing bacteria, mainly strains already described, was made to determine the range of alkenes (C_2-C_6) and alkanes (C_1-C_6) used for growth, the substrate specificity of alkene-grown bacteria and the formation and excretion of epoxides by washed cells.

Growth on alkenes and alkanes.

The eleven strains of alkene-utilizing bacteria investigated grew on only one or two alkenes, except Nocardia H8 which could utilize all 1-alkenes tested (Table 2). A certain common pattern in alkene utilization was observed. For instance, all propene utilizers also grew on 1-butene and all 1,3-butadiene-utilizing Nocardia spp. grew on isoprene. Although only Myco-

bacterium spp. were isolated with ethene as carbon and energy source (Table 1), bacteria of other genera were able to metabolize this compound as well (Table 2).

Gaseous alkenes are produced naturally and chemically in large amounts but the concentrations in nature are mostly very low (<5 vpb) (1,31). Although growth on alkenes at these concentrations may be possible, it is very likely that other com-

Table 1. Micro-organisms isolated from soil samples from 10 different locations on various gaseous and volatile alkenes. The number of strains isolated is shown between brackets.

Alkene	Isolates
Ethene	Mycobacterium (10)
Propene	Xanthobacter (9), Mycobacterium (1)
1-Butene	Xanthobacter (8), Nocardia (1), Mycobacterium (1)
2-Butene	Nocardia (2), Mycobacterium (1)
1,3-Butadiene	Nocardia (10)
Isoprene	Nocardia (10)
1-Hexene	Pseudomonas (9), Nocardia (1)

Table 2. Growth of several alkene-utilizing bacteria on ethene, propene, 1-butene, 1-pentene, 1-hexene, 1,3-butadiene, isoprene and 2-butene, respectively.

Substrate	C_2H_4	C_3H_6	C_4H_8	C_5H_{10}	C_6H_{12}	C_4H_6	C_5H_8	C_4H_8
Strain								
Mycobacterium E3	+	-	-	-	-	-	-	-
Mycobacterium 2W	+	-	-	-	-	-	-	-
Mycobacterium Py1	-	+	+	-	-	-	-	-
Xanthobacter Py2	+	+	+	-	-	+	-	-
Nocardia By1	-	+	+	-	-	-	-	-
Xanthobacter By2	+	+	+	-	-	+	-	-
Nocardia TB1	-	-	-	-	-	-	-	+
Nocardia BT1	-	-	-	-	-	+	+	-
Nocardia IP1	-	-	-	-	-	+	+	-
Pseudomonas H1	-	-	-	-	+	-	-	-
Nocardia H8	+	+	+	+	+	-	-	-

Table 3. Growth of several gaseous and volatile alkene-utilizing bacteria on monoterpenes.

Bacterium	Myrcene	Limonene	γ-Terpinene	β-Pinene	α-Pinene
Mycobacterium E3	+	-	-	-	+
Mycobacterium 2W	+	-	-	-	-
Mycobacterium Py1	+	+	+	+	+
Xanthobacter Py2	-	-	-	-	-
Nocardia By1	+	-	-	-	-
Xanthobacter By2	-	-	-	-	-
Nocardia TB1	+	+	+	+	+
Nocardia BT1	-	-	-	-	+
Nocardia IP1	-	+	-	+	+
Pseudomonas H1	-	-	-	-	-
Nocardia H8	+	+	+	+	+

pounds are used as carbon and energy source. A broad range of non-hydrocarbons as for instance alcohols, aldehydes, organic acids and carbohydrates also supported growth of alkene-utilizing bacteria explaining the presence of these organisms in the ecosystems analyzed. The capacity to degrade gaseous alkenes may originate from the ability of these organisms to grow also on alkenes that are present in higher concentrations in nature, e.g. monoterpenes. For this reason we have investigated the ability of alkene-utilizing bacteria to grow on these branched alkenes. Xanthobacter spp. and Pseudomonas H1 were not able to grow on any monoterpene tested, but all other alkene-utilizing bacteria were able to grow on several monoterpenes. Mycobacterium Py1, Nocardia TB1 and Nocardia H8 even utilized all monoterpenes tested. The non-cyclic monoterpenes, myrcene and α-pinene supported growth of six micro-organisms tested (Table 3).

Some of the alkene-utilizing bacteria also grew on a limited number of saturated hydrocarbons. Pseudomonas H1 was able to grow on pentane and hexane whereas Nocardia TB1 also grew on these hydrocarbons as well as on propane and butane. Mycobacterium 2W and Mycobacterium Py1 grew on hexane while Nocardia BT1 and Nocardia IP1 possessed the ability to utilize propane and butane as sole source of carbon and energy.

Oxidation of alkanes.

Alkene-grown bacteria investigated up to now generally were not able to hydroxylate alkanes, whereas alkane-grown bacteria were able to hydroxylate alkanes as well as to epoxidate alke-

nes. Resting-cell suspensions of the eleven alkene-grown bacteria were examined for their ability to oxidize gaseous alkanes, pentane and hexane. Interestingly, three of the eleven strains tested oxidized alkanes when grown on alkenes. Isoprene-grown Nocardia IP1 oxidized butane, pentane and hexane at rates up to 2 nmol per minute per mg protein. Trans-2-butene-grown Nocardia TB1 oxidized all alkanes tested except methane and the oxidation rates were comparable to the oxidation rates of isoprene-grown Nocardia IP1 except that the rate of oxidation of butane was twice as high. 1-Hexene-grown Pseudomonas H1 oxidized pentane and hexane at rates of 4 to 6 nmol per minute per mg protein, whereas the gaseous alkanes were only oxidized at negligible rates. Other alkene-grown bacteria tested were not able to oxidize gaseous or volatile alkanes.

Oxidation of alkenes.

All alkene-grown bacteria tested oxidized the gaseous and volatile alkenes used. In general, highest alkene oxidation rates were found with the alkene on which the bacterium was grown (Table 4). In this respect, 1-hexene-grown Nocardia H8 is exceptional, because all 1-alkenes were oxidized at the same

Table 4. Oxidation of ethene, propene, 1-butene, 1,3-butadiene, cis-2-butene, trans-2-butene, 1-pentene and 1-hexene, respectively, by washed cell suspensions of alkene-grown bacteria.

	Substrate	C_2H_4	C_3H_6	C_4H_8	C_4H_6	C_4H_8 cis	C_4H_8 trans	C_5H_{10}	C_6H_{12}
Strain	Growth substrate	Substrate oxidation rate[1]							
Mycobacterium E3	Ethene	50	17	12	19	20	20	14	13
Mycobacterium 2W	Ethene	23	6	6	11	12	13	6	7
Mycobacterium Py1	Propene	15	20	17	-2	-	-	8	5
Xanthobacter Py2	Propene	50	81	62	17	70	60	20	14
Nocardia By1	1-Butene	19	23	26	9	21	17	12	11
Xanthobacter By2	1-Butene	45	70	61	24	67	29	24	16
Nocardia TB1	2-Butene	2	2	3	3	6	5	1	1
Nocardia BT1	Butadiene	18	16	17	57	19	19	-	-
Nocardia IP1	Isoprene	11	14	12	13	12	15	-	-
Pseudomonas H1	1-Hexene	0	0	1	1	1	1	3	5
Nocardia H8	1-Hexene	16	19	19	-	-	-	16	16
Nocardia H8	Propene	17	18	16	-	-	-	17	16

1 in nmol min^{-1} (mg protein)$^{-1}$

2 not determined

rate and when grown on another alkene similar oxidation rates were found. Oxidation rates of 1-alkenes by Nocardia TB1 and Pseudomonas H1 were low and after a short period of time the activity of the washed cell suspensions levelled off, whereas alkanes were oxidized to completion by trans-2-butene-grown Nocardia TB1 (Fig. 1) and Pseudomonas H1. All other alkene-grown bacteria oxidized the unsaturated hydrocarbons at rates of 10 to 80 nmol per minute per mg protein (Table 4) and no decrease in activity was detected during the time course of the experiment. The oxidation of alkenes by non-growing cells may either result in a complete oxidation to CO_2 and H_2O or in the accumulation of partially oxidized products.

Table 5. Formation of epoxyalkanes from ethene, propene, 1-butene, cis-2-butene, trans-2-butene, respectively, by washed cell suspensions of alkene-grown bacteria.

	Product	C_2H_4O	C_3H_6O	C_4H_8O	C_4H_8O cis	C_4H_8O trans
Strain	Growth substrate	Product fomation rate[1]				
Mycobacterium E3·	Ethene	0	15	11	20	20
Mycobacterium 2W	Ethene	0	5	4	12	13
Mycobacterium Py1	Propene	15	0	0	-2	-
Xanthobacter Py2	Propene	46	0	0	17	41
Nocardia By1	1-Butene	9	0	0	7	11
Xanthobacter By2	1-Butene	11	0	0	27	22
Nocardia TB1	2-Butene	2	2	2	0	0
Nocardia BT1	Butadiene	10	13	14	19	19
Nocardia IP1	Isoprene	2	9	6	7	6
Nocardia H8	1-Hexene	0	0	0	-	-
Nocardia H8	Propene	0	0	0	-	-

1 in nmol min^{-1} (mg protein)$^{-1}$

2 not determined

None of the strains tested excreted 1,2-epoxy-3-butene from 1,3-butadiene. Both ethene-grown mycobacteria excreted 1,2-epoxypentane and 1,2-epoxy-hexane from the respective 1-alkenes at rates up to 2 nmol per minute per mg protein.

Formation of epoxyalkanes.

Washed cell suspensions of the eleven strains of alkene-utilizing bacteria were able to accumulate epoxyalkanes from

one or more alkenes. However, no significant excretion of epoxyalkanes was detected from alkenes on which the bacteria were grown (Table 5). Epoxyalkanes also did not accumulate from alkenes which were potential growth substrates of the bacteria. For instance, Nocardia H8 able to grow on all 1-alkenes tested did not form any 1,2-epoxyalkane. 1,2-Epoxyethane formation by alkene-grown Xanthobacter spp. was an exception because these bacteria were able to grow on ethene at very slow rates (12). No formation of epoxides was observed from 1,3-butadiene by alkene-grown bacteria. Formation of 1,2-epoxypentane and 1,2-epoxyhexane was demonstrated with Mycobacterium E3 and Mycobacterium 2W. The epoxyalkane formation rates varied from 1 to 50 nmol per minute per mg protein and highest rates were found with ethene- and propene-grown bacteria (Table 5). When comparing alkene oxidation rates from Table 4 and epoxyalkane formation rates from Table 5, it is obvious that epoxides were formed stoichiometrically in only a few cases. 2,3-Epoxybutanes were not degraded by ethene-grown Mycobacterium spp. and 1,3-butadiene-grown Nocardia BT1 and consequently accumulated

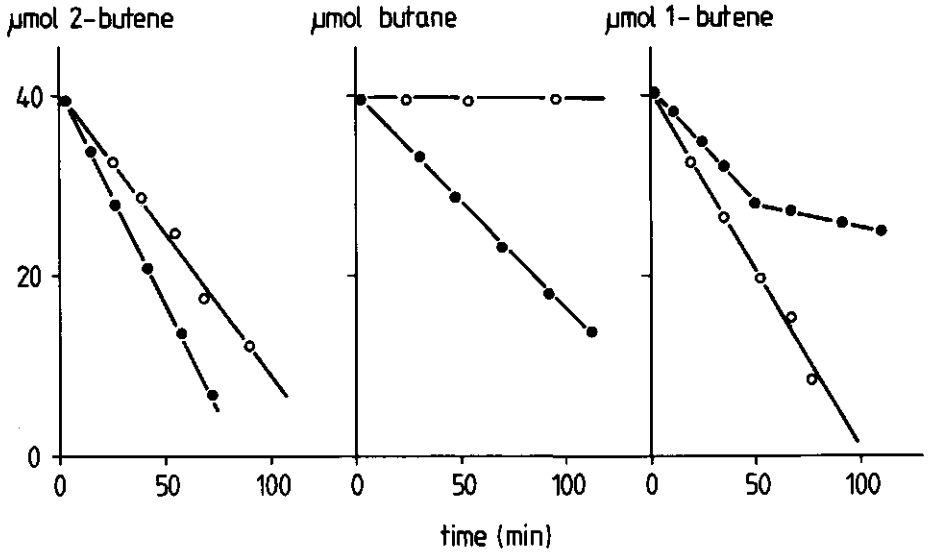

Figure 1. Oxidation of C_4 hydrocarbons by washed cell suspensions of Nocardia By1 grown on 1-butene (○) and Nocardia TB1 grown on 2-butene (●).

stoichiometrically in the supernatant. Nocardia TB1 formed 1,2-epoxyalkanes only stoichiometrically from ethene and propene. Finally, Mycobacterium Py1 did not degrade 1,2-epoxyethane. However, from most non-growth alkenes, only a portion of the alkene oxidized by the alkene-grown bacteria was recovered as epoxyalkane during the time course of the experiment.

Utilization of epoxyalkanes.

Alkene-utilizing bacteria are able to oxidize some epoxyalkanes because these compounds are intermediates in the degradative pathways of alkenes (4,7,13). Most epoxyalkanes originating from non-growth alkenes were also degraded by alkene-grown bacteria. In order to determine whether epoxyalkanes originating from these non-growth alkenes were oxidized to CO_2 and H_2O or converted to another product, the degradation of 1,2-epoxypropane by washed cell suspensions of ethene-grown Mycobacterium E3, was investigated. During the degradation of 1,2-epoxypropane by ethene-grown Mycobacterium E3 no additional CO_2 was formed over the CO_2 formed endogenously. Subsequently, it was shown that 1,2-epoxypropane was hydrolysed to 1,2-propanediol by washed cell suspensions of ethene-grown Mycobacterium E3 (Fig. 2).

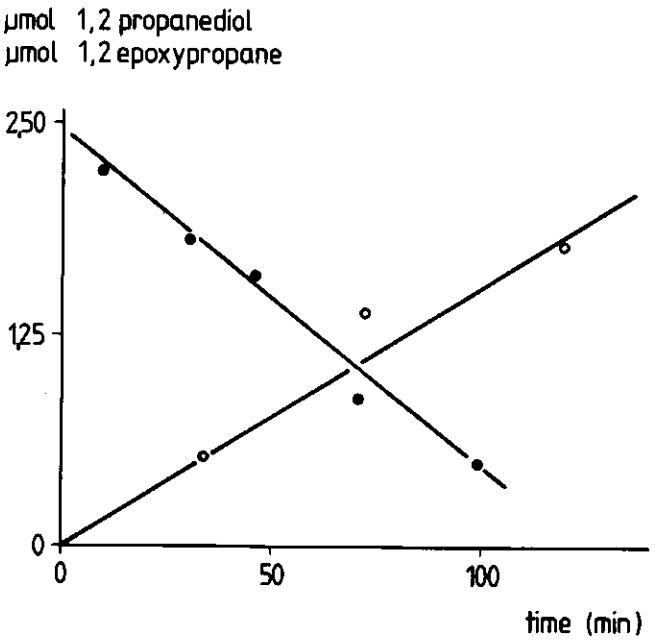

Figure 2. The formation of 1,2-propanediol (○) from 1,2-epoxypropane (●) by washed cell suspensions of ethene-grown Mycobacterium E3 (9.5 mg protein).

DISCUSSION

Gaseous and volatile alkenes can serve as sole carbon and energy sources for microbial growth. Especially, taxonomically related Gram-positive bacteria have been isolated using these compounds as a growth substrate (Table 1). Gram-variable Xanthobacter spp. may seem an exception but these bacteria show some resemblance with Corynebacterium spp. (38). The isolation of Gram-positive bacteria on alkenes by others (5,10,11,19,39) is in agreement with these results. Gram-negative Pseudomonas spp. were isolated using enrichment cultures with 1-hexene as the sole carbon and energy source. This alkene is probably a borderline case between lower gaseous and higher liquid alkenes. Pseudomonas spp. have been described as liquid 1-alkene utilizers (25,32). From Table 2 it is obvious that most alkene-utilizing bacteria can grow on only a limited number of alkenes. A relatively wide range of alkenes supported growth of Xanthobacter spp. while Nocardia H8 seems to be exceptional since it utilized all 1-alkenes tested. Until recently, only Mycobacterium spp. tested were known to grow on ethene but it is clear now that Xanthobacter spp. and Nocardia spp. can also grow on this naturally occuring plant hormone (Table 2).

Only few bacteria that have been isolated on gaseous alkenes can also grow on saturated gaseous hydrocarbons namely Nocardia corallina B276 (11), Mycobacterium E20 (4) and other Mycobacterium spp. (10). Furthermore, only a very limited number of gaseous alkane-utilizing bacteria could grow on gaseous alkenes (9,26). In general, these observations have now been confirmed by the results obtained with the alkene-utilizing bacteria selected because only three strains were capable of growth on some gaseous alkanes. Especially, 1-hexene-utilizing Pseudomonas H1 and trans-2-butene-utilizing Nocardia TB1 grew more abundantly on some alkanes than on alkenes and additional experiments showed that both bacteria more resemble alkane-utilizing bacteria than their alkene-utilizing counterparts.

In view of the low alkene concentrations in nature, it is likely that alkene-utilizing bacteria also utilize other naturally occurring carbon and energy sources to sustain live. The ability to utilize gaseous alkenes might have evolved from the potential to degrade saturated hydrocarbons present more abundantly in nature. As stated above Nocardia TB1 and Pseudomonas H1 are better described as alkane-utilizers and for these oganisms saturated hydrocarbons may be a more important substrate in nature. The high incidence of monoterpene utilization by Mycobacterium and Nocardia strains suggests that these bacteria may use these naturally occurring compounds as carbon and energy source in soil ecosystems. Alkene-utilizing

Xanthobacter strains, however, were unable to grow on monoterpenes so these organisms will have to rely on other naturally occurring compounds.

Oxidation of both alkenes and alkanes by hydrocarbon-grown micro-organisms occurs frequently. Hydrocarbon mono-oxygenases derived from methane- and n-alkane-grown micro-organisms hydroxylate gaseous and volatile alkanes and epoxidize alkenes (20,21,23,29,33), whereas alkene mono-oxygenase derived from ethene-, propene- and 1,3-butadiene-grown cells only epoxidize alkenes (4,12,16).

Some of the selected strains grown on alkenes were able to oxidize both saturated and unsaturated hydrocarbons and one therefore can assume that an alkane type mono-oxygenase is present in these cells. Indeed Pseudomonas H1 is a bacterium which resembles a Pseudomonas strain used by Thijsse and van der Linden (35) and it was shown by these authors that the initial attack on 1-hexene is preponderantly via the methyl group. Nocardia TB1 grows more abundantly on saturated than on unsaturated gaseous hydrocarbons and evidence will be presented elswhere that Nocardia TB1 metabolizes trans-2-butene via crotonic alcohol (14).

Resting-cell suspensions of bacteria grown on alkenes readily oxidize all alkenes and alkadienes tested (Table 3) and this suggests that the mono-oxygenases involved do not have a high degree of specificity towards alkenes. Such a broad substrate specificity towards hydrocarbons is not unique because alkane-grown bacteria act in the same way (20,21,23,29, 33). However, 1-hexene-grown Pseudomonas is an exception because this bacterium did not oxidize the gaseous alkenes to any significant extent.

Although Pseudomonas H1 and Nocardia TB1 oxidize 1-alkenes, the oxidizing activity levelled off after a short period of time. This decreasing activity is probably due to the toxic effect of the epoxyalkanes formed and such susceptibility towards epoxides is found in alkane-utilizing bacteria but not in alkene-utilizers (17). Nocardia TB1 and Pseudomonas H1 also in this respect resemble the alkane utilizers. The general differences observed between alkane- and alkene-utilizers with respect to both substrate specificity and epoxide toxicity are illustrated in Fig. 1. In this figure activities towards C_4 hydrocarbons of washed cells of 1-alkene-grown Nocardia cells are compared with these activities of 2-butene-grown Nocardia cells.

It is obvious that no epoxides accumulated from alkenes on which the bacterium was grown. However, alkene-grown bacteria, in general, accumulate epoxyalkanes from non-growth alkenes. This epoxide formation by alkene-utilizing bacteria is a con-

sequence of the restrictive range of epoxides converted by 1,2-epoxyalkane degrading enzymes (16). Stoichiometric formation of epoxyalkane from 2-butene was found with ethene-grown Mycobacterium spp. and with 1,3-butadiene-grown Nocardia BT1, whereas propene-grown Mycobacterium Py1 cells accumulated stoichiometric amounts of 1,2-epoxyethane from ethene (7). Both Nocardia TB1 and Pseudomonas H1 do not metabolize 1,2-epoxyalkanes or oxidize epoxyalkanes at negligible rates and in this respect these bacteria again act in the same way as alkane-utilizing bacteria (29).

Nevertheless, most epoxyalkanes derived from non-growth alkenes are degraded further by alkene-utilizing bacteria. Ethene-grown Mycobacterium E3 catalysed the hydrolysis of 1,2-epoxypropane to 1,2-propanediol but 1,2-propanediol formation by Mycobacterium E3 is probably not mediated by an enzyme of the degradative pathway of ethene (87). Such diol formation from epoxyalkanes is not catalysed by methane-grown bacteria either (22) and information about this reaction in bacteria is scarce (6).

The formation rates of epoxyalkanes in ethene- and propene-grown bacteria are of the same order as the reported activities in methane- and gaseous alkane-utilizing bacteria (21,23,29, 33). The lower activities in other alkene-grown bacteria are probably due to the molecular size of the substrate because to support growth less substrate has to be oxygenated by the monooxygenases responsible.

Finally, from the results presented it is obvious that almost every epoxyalkane may be formed by alkene-grown bacteria when an appropriate combination of bacterium and alkene is used.

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CHAPTER 8

ETHYLENE OXIDE PRODUCTION BY IMMOBILIZED MYCOBACTERIUM Py1 IN A GAS-SOLID BIOREACTOR

J.A.M. de Bont, C.G. van Ginkel, J. Tramper and K.Ch.A.M. Luyben

SUMMARY

The propylene-utilizing Mycobacterium Py1 has been shown to oxidize gaseous alkenes, including ethylene, to the corresponding epoxides through a NAD(P)H-requiring monooxygenase. The organism could not metabolize ethylene oxide and as a consequence excreted this epoxide when fed with ethylene. Prolonged formation of ethylene oxide by cells immobilized in alginate gel or fixed on sand was dependent on a cosubstrate for regeneration of NAD(P)H used in the monooxygenase reaction. Experiments were performed in a gas-solid reactor to prevent accumulation of the toxic ethylene oxide in the immediate vicinity of the biocatalyst.

Enzyme Microb Technol 1983 5:55-59

INTRODUCTION

The ability of microorganisms to grow on gaseous alkenes like ethylene and propylene has only been detected in a few cases to date (de Bont, 1976; Heyer, 1976; Cerniglia et al., 1976; de Bont et al., 1980; Furuhashi et al., 1981). The oxidation of these and other unsaturated hydrocarbons has been studied mainly with organisms isolated and grown on saturated hydrocarbons. Although such alkane-utilizing organisms are generally not able to grow on alkenes, they nevertheless are able to oxidize these compounds either by hydroxylation of a methyl group or, more commonly, by the epoxidation of the double bond (Huybregtse and van der Linden, 1963; Thijsse and van der Linden, 1963; May et al., 1975; Markovetz et al., 1967; Cardini and Jurtshuk, 1968; Abbott and Hou, 1973). Cooxidation of gaseous alkanes and alkenes has also long been known to occur in methane-utilizing bacteria. These organisms can be grown only on a restricted range of reduced C_1-compounds but may partially oxidize ethane, propane and butane (Foster and Davis, 1966), ethylene (de Bont and Mulder, 1974), propylene and many other compounds (Tonge et al., 1977; Colby et al., 1977; Patel et al., 1978; Stirling and Dalton, 1979; Hou et al., 1979; Higgins et al., 1979). With the exception of Pseudomonas oleovorans (May et al., 1975), oxidation of gaseous alkenes by alkane- and alkene-utilizing micro-organisms results in the formation of the corresponding epoxides. In preliminary experiments with the propylene-utilizing Mycobacterium Py1 (de Bont et al., 1980) it was also observed that this organism was able to excrete certain epoxides from alkenes.

In recent years, this epoxidation of alkenes by bacteria has received considerable attention because of a possible production of certain epoxides by means of a biotechnological process (Furuhashi et al., 1981; Higgins et al., 1980; de Bont et al., 1981). A method employing immobilized enzymes has been developed (Parkinson, 1980), but manufacture of epoxides using immobilized whole cells seems more feasible. Not only can enzyme-isolation procedures be omitted, but co-factor regeneration is more easily achieved.

A major problem in the biotechnological process is the toxicity of the reaction product for the biocatalyst. To avoid epoxide accumulation in the microenvironment of the biocatalyst, and thus inactivation, rapid removal of this product is essential.

The propylene-utilizing Mycobacterium Py1 used in this investigation produces ethylene oxide from ethylene. To promote a rapid and continuous removal of the toxic ethylene oxide from

the microenvironment of the immobilized cells, a small gas-solid bioreactor was used for the production of this epoxide on a small scale.

MATERIALS AND METHODS

Mycobacterium Py1 (de Bont et al. 1980) used in this study was maintained and cultivated like the ethylene-utilizing bacterium E44 (Wiegant and de Bont, 1980) except that ethylene was replaced by propylene. Preparation of suspensions of washed cells and cell-free extracts, and gas chromatographic determinations of gaseous hydrocarbons, epoxides, aldehydes and glycols have been described previously (Wiegant and de Bont, 1980; de Bont et al., 1979). Carbon dioxide was determined with a Becker 406 gas chromatograph fitted with a Porapak Q column; column temperature was 50°C and argon was the carrier gas.

Metabolic activities of washed cell suspensions

Oxidation of hydrocarbons and epoxides, and excretion of epoxides by washed cell suspensions (13 cm^3 final volume) were measured by incubating cells at 30°C in 50 mM potassium phosphate buffer, pH 7.2, in a vessel sealed with a Yellow Springs Instruments model 53 oxygen sensor. No gas phase was present in the vessel and the oxygen sensor prevented gas exchange with the atmosphere. The oxygen sensor served to check aerobic conditions (excess oxygen) during the periods of incubation. Substrates and methane (internal standard) were given as aqueous solutions. Mixing in the vessels was provided by a magnetic stirrer. Samples (2 cm^3) from the incubation mixture were periodically withdrawn by syringe and injected immediately into Hungate tubes (17 cm^3) kept in a boiling water bath. Concentrations of hydrocarbons and epoxides were determined gas chromatographically by taking samples from the gas phase of the Hungate tubes.

Immobilization procedures

(1) Five cm^3 of a cell suspension (80 kg protein m^{-3}) in 50 mM potassium phosphate buffer, pH 7.2, was mixed with 20 cm^3 of an aqueous solution of sodium alginate (20 kg m^{-3}, Manucol DM, Alginate Industries London) and sodium chloride (9 kg m^{-3}). The degassed mixture was extruded into a 0.1 M $CaCl_2$ solution. The size of the drops formed at the needle tip was adjusted by a longitudinal airflow. The resulting beads had a diameter of $\pm$ 1.2 mm.
(2) Local sand was extensively washed with water and then dried overnight at 100°C. Forty grams of the cooled sand was thoroughly mixed with 5 cm^3 of a cell suspension (80 kg protein m^3) in 50 mM potassium phosphate buffer, pH 7.2, and placed in the bioreactor.

Gas-solid bioreactor

The gas-solid bioreactor system (Figure 2) consisted of the solid, immobilized biocatalyst contained in a tubular reactor, an absorber, and a pump-in-line. In this closed bioreactor system, the gaseous substrates and the co-substrates were injected together with the reference gas (methane) and recirculated. The reaction was performed batchwise and care was taken that

the volume of the gas phase (1.5 dm^3) was sufficiently large that excess oxygen was assured. In the absorber, which contained water acidified to 1.5 with sulphuric acid, the epoxides produced were extracted with concomittant hydrolysis to glycols, and analysed as such. For that, samples of the aqueous phase were taken at regular intervals. Similarly, samples of the gas phase were taken in the top section of the bioreactor for the analysis of alkenes and alkene oxides. All the reactions were performed at 25°C.

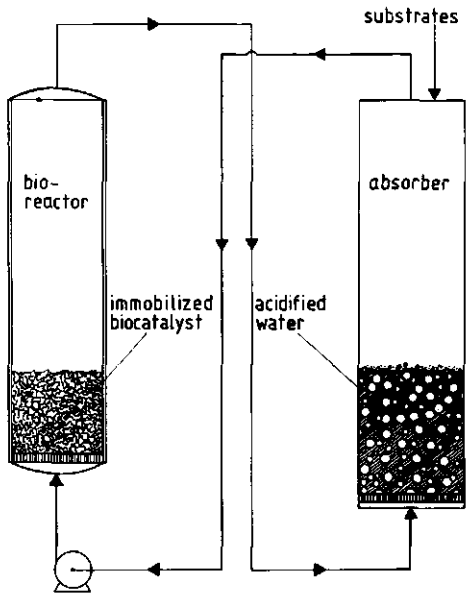

Figure 2. Schematic representation of the bioreactor system for the production of ethylene oxide with Mycobacterium Py1 immobilized on sand or in alginate beads.

RESULTS

Growth media

Mycobacterium Py1 grew on propylene and butylene with culture doubling times of 27 and 40 h, respectively, and on a wide variety of other carbon and energy sources, such as ethanol, D-glucose and succinate. No growth occurred, however, when ethylene was used as both the carbon and energy source.

Metabolism of propylene via propylene oxide

Metabolism of propylene by Mycobacterium Py1 involves propylene oxide as an intermediate since washed suspensions of propylene-grown cells excreted traces of this epoxide when incubated in the presence of propylene. Propylene oxide could

Table 1. Oxidation of propylene and excretion of propylene oxide from propylene by washed cell suspensions of Mycobacterium Py1 as effected by 10 mM butylene oxide.

	Propylene oxidized (mmol min-1 kg-1 protein)	Propylene oxide excreted (mmol min-1 kg-1 protein)
Without butylene oxide	40	2.5
With 10 mM butylene oxide	9.5	9.5

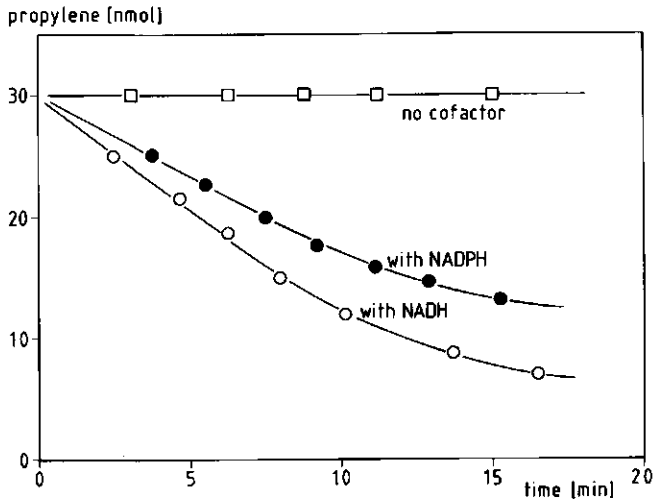

Figure 1. Oxidation of propylene by cell-free extracts (1.8 mg protein) of Mycobacterium Py1 grown on propylene without cofactor (□), in the presence of NADH (○) or NADPH (●).

be recovered quantitatively from propylene in the presence of butylene oxide (Table 1). In a separate experiment, aimed at elucidating this behaviour, it was demonstrated that butylene oxide at a concentration of 10 mM completely inhibited the oxidation of propylene oxide fed to washed cells.

Involvement of a monooxygenase

To establish the nature of the enzyme system involved in the conversion of propylene into propylene oxide, the disappearance of propylene was also studied *in vitro* using cell-free extracts. Oxidation of propylene by extracts was dependent on either NADH or NADPH (Figure 1). No activity was observed with NADH in the absence of oxygen, with ascorbate replacing NAD(P)H, or with boiled extract. These results indicate that propylene is oxidized in *Mycobacterium* Py1 by a monooxygenase.

Substrate specificity

The substrate specificity of the propylene monooxygenase towards some gaseous hydrocarbons was determined using whole-cell suspensions of propylene-grown cells. Similar cell suspensions were used to examine the substrate specificity of the enzyme involved in the further metabolism of propylene oxide. The results given in Table 2 show that propylene-grown cells

Table 2. Oxidation of gaseous hydrocarbons and epoxides by washed cell suspensions of propylene-grown *Mycobacterium* Py1.

Substrate	Conversion rate mmol min^{-1} $(kg\ protein)^{-1}$
Ethylene	50
Propylene	45
Butylene	25
Butadiene	70
Allene	85
Ethane	0
Propane	0
Butane	0
Ethylene oxide	0
Propylene oxide	25
Butylene oxide	12

can also oxidize other alkenes including ethylene, but not

alkanes. Most interestingly, the enzyme involved in the further metabolism of propylene oxide did not oxidize ethylene oxide.

Using cell-free extracts of propylene-grown cells it was confirmed that ethylene was oxidized by the propylene monooxygenase since the reaction with ethylene was strictly NAD(P)H-dependent. The reaction product from ethylene and oxygen was ethylene oxide.

Ethylene oxide from ethylene and oxygen

The ability of Mycobacterium Py1 to oxidize ethylene, combined with its inability further to metabolize ethylene oxide, could be made use of to form ethylene oxide from ethylene and oxygen by whole cells. Washed cell suspensions of propylene-grown cells quantitatively produced ethylene oxide from ethylene.

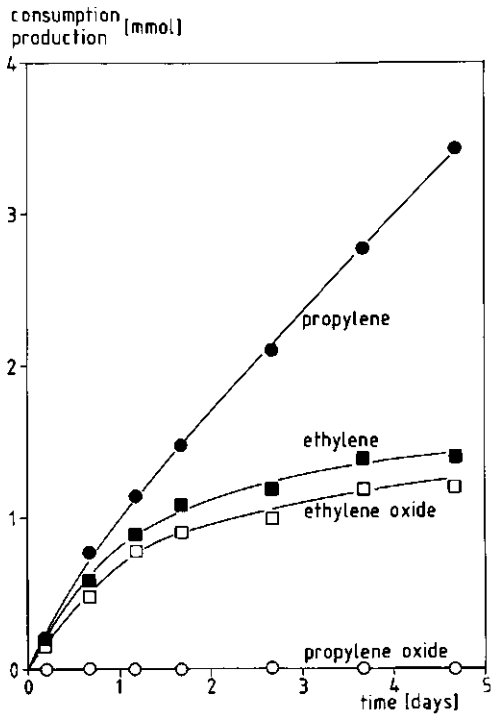

Figure 3. Oxidation of propylene and conversion of ethylene into ethylene oxide by cells of propylene-grown Mycobacterium Py1 immobilized in alginate gel. Oxidation rates by immobilized cells (each incubation 80 mg protein) were measured in two gas-solid reactors with propylene (●) and ethylene (■) respectively. Propylene oxide (○) and ethylene oxide (□) were determined as the corresponding glycols. For details, see Figure 2.

Gas-solid bioreactor

The potential of Mycobacterium Py1 to produce ethylene oxide was further investigated with propylene-grown cells that were either immobilized in alginate gel or fixed on sand. These immobilized cells were examined in a small gas-solid reactor (Figure 2). Operation of this reactor allowed a swift and continuous removal of ethylene oxide from the biocatalyst: ethylene oxide and other epoxides are toxic for biological materials. For Mycobacterium Py1, immobilized on sand and kept in screw-cap bottles in the presence of 100 mM ethylene oxide, the oxidation rate of propylene was reduced to 40% of the original rate.

The rate of propylene oxidation by alginate-immobilized cells remained almost constant during 5 days, but the ethylene oxidation rate levelled off after 1 to 2 days of operation (Figure 3). No propylene oxide accumulated from propylene, whereas ethylene oxide was recovered almost quantitatively from ethylene.

Cofactor regeneration

During the oxidation of ethylene or propylene to the corresponding epoxides by the immobilized cells a continuous regeneration of NAD(P)H from NAD(P) is essential since the formation of the epoxide is brought about by an NAD(P)H-dependent monooxygenase. With propylene as substrate, this cofactor regeneration is apparently possible by the further oxidation of propylene oxide by the immobilized cells since no accumulation of propylene oxide occurred and only a minor decrease in activity was observed in 5 days (Figure 3). Ethylene oxide, however, is not metabolized further and thus the reduction of NAD(P), when ethylene is given as substrate to the cells, is only possible through endogenous respiration or through the oxidation of substrates that are supplied additionally to the cells. Endogenous respiration of cells, fixed on sand, as effected by either ethylene or ethylene oxide was investigated by following the carbon dioxide evolution from the cells as influenced by these two compounds. Ethylene oxide was not oxidized by the cells and ethylene was quantitatively converted into ethylene oxide (Figure 4a). Ethylene-derived carbon was therefore not oxidized to CO_2 but, nevertheless production of CO_2 by the immobilized cells was enhanced by ethylene (Figure 4b). Since ethylene oxide did not stimulate CO_2 production, it can be concluded that the NAD(P)H required to form ethylene oxide from ethylene was regenerated by the immobilized cells via an increase in the rate of endogenous respiration.

Addition of an external cosubstrate which may be oxidized by the whole cells may also supply the cells with the NAD(P)H

required for the ethylene oxidation. Several substrates may be suitable as cosubstrate for NAD(P)H regeneration. In the present investigation, propionaldehyde was chosen as cosubstrate because the volatile aldehyde could conveniently be employed in the bioreactor system and since Mycobacterium Py1 contains a NAD-dependent propionaldehyde dehydrogenase. It was found that the oxidation of ethylene by cells immobilized on sand was promoted by supplying propionaldehyde to the cells in the bioreactor (Figure 5), indicating a partial regeneration of NAD(P)H by means of the cosubstrate propionaldehyde.

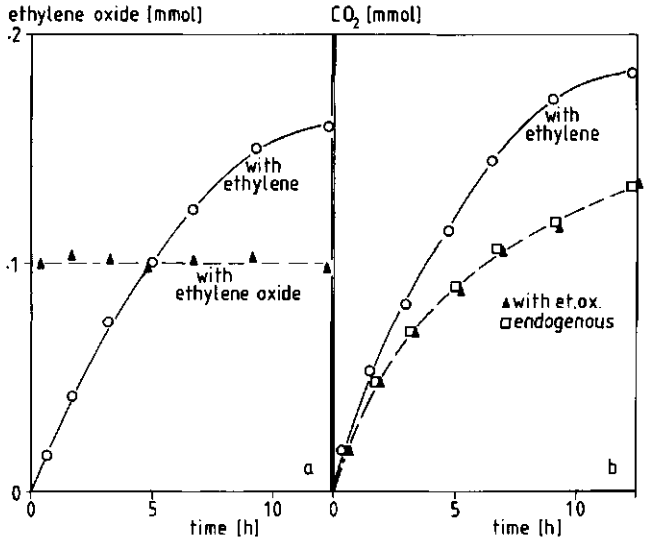

Figure 4. (a) Effect of propylene-grown immobilized cells of Mycobacterium Py1 on ethylene and ethylene oxide and (b) evolution of carbon dioxide from these cells as influenced by ethylene and ethylene oxide. Three screw-cap bottles (70 cm^3) received each cells (30 mg protein) fixed on sand (20 g). Ethylene was injected into one bottle and the formation of ethylene oxide and carbon dioxide (○) were followed. Into the second bottle (▲) ethylene oxide was injected while the third bottle (□) served as a control.

DISCUSSION

The propylene monooxygenase of Mycobacterium Py1 resembles the alkene monooxygenase of the ethylene-utilizing Mycobacterium E20 (de Bont et al., 1979) in that it oxidizes various alkenes to epoxides while alkanes are not oxidized. This substrate specificity is unusual because most of the alkene-

epoxidating enzymes described to date also hydroxylate alkanes to the corresponding alcohols.

The further metabolism of propylene oxide by Mycobacterium Py1 has not been investigated. Epoxide metabolism by the organism might possibly involve hydrolysis of the epoxide to 1,2-propanediol as found in a propylene oxide-utilizing Nocardia sp. (de Bont et al., 1982). Alternatively, an enzyme resembling ethylene oxide dehydrogenase, as reported for the ethylene-utilizing bacteria (de Bont and Harder, 1978; Wiegant and de Bont, 1980), may be present in Mycobacterium Py1. Oxidation of ethylene by propylene monooxygenase in combination with the lack of activity of the propylene oxide-degrading enzyme towards ethylene oxide resulted in the excretion of the latter epoxide by both washed cell suspensions and immobilized cells. Both immobilization on sand and in alginate yielded

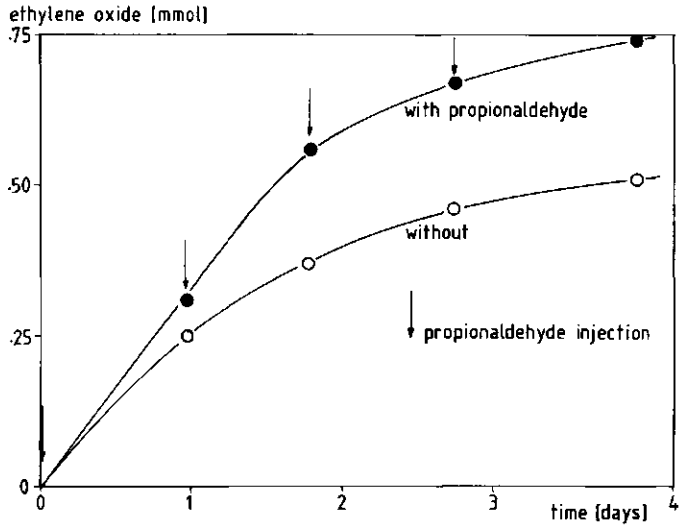

Figure 5. Ethylene oxide formation from ethylene by cells of propylene-grown Mycobacterium Py1 immobilized on sand. Epoxide formation by the immobilized cells (32 mg protein for each incubation) was followed in two gas-solid reactors in the presence (●) or absence (○) of propionaldehyde. This cosubstrate was injected (0.25 mmol each time) into the acidified water of the absorber at the moments indicated by the arrows. Ethylene oxide was determined as ethylene glycol. For details see Figure 2.

active biocatalyst preparations, with retention of 60 to 30% of the activity, respectively. However, both preparations eventually produced the same amount of ethylene oxide because of

the longer half-life of the biocatalyst immobilized in alginate. It is likely that the rate of oxidation is reduced by diffusion limitation of the substrate in the latter system, while the amount of substrate that can be oxidized is determined by available cofactor.

A problem associated with epoxide formation employing a monooxygenase catalysed reaction is the need to regenerate NAD(P)H from NAD(P). Reduction of this cofactor occurred to a certain extent at the expense of an elevated endogenous respiration, but supply of the immobilized cells with an external cosubstrate is necessary for prolonged biocatalysis. In the present investigation, propionaldehyde was used to demonstrate an effect of a cosubstrate on epoxide formation by the immobilized cells. Other compounds may be better candidates to serve as cosubstrate. This will be the subject of another study.

A new type of bioreactor was used in the present investigation to overcome toxicity effects of the reaction product on the immobilized biocatalyst. Ethylene oxide is very soluble in water and no suitable extractant, immiscible with water is available. Therefore, the approach of using a multiliquid/gas/solid bioreactor we have proposed (de Bont et al., 1981) for the production of propylene oxide, is not feasible in this case. However, by using a gas-solid reactor system (Figure 2), it is possible to operate at an optimal substrate concentration since quantitative conversion can be established by recirculation of the gas phase. Recirculation at the same time makes possible a continuous removal of the toxic product in the absorber, thus keeping the concentration to a minimum in the immediate vicinity of the immobilized biocatalyst. Saturation of the gas phase with water prevents the biocatalyst from drying out and concomittant inactivation.

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CHAPTER 9

REMOVAL OF ETHENE TO VERY LOW CONCENTRATIONS BY IMMOBILIZED MYCOBACTERIUM E3

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H.A.M. Boerrigter

SUMMARY

Removal of ethene to concentrations below 1 vpm from the air in storage facilities is necessary to prevent the deterioration of stored fruits, vegetables and flowers. Ethene-utilizing Mycobacterium E3 organisms are able to oxidize ethene to these low concentrations. Ethene was oxidized at the same rate by organisms immobilized on lava or perlite as it was by the suspension of cells. The residence times in a gas/solid bioreactor necessary to convert 66% of the ethene with an initial concentration of 3.2 vpm, and with cell loads on lava of 0.72 or 0.36 mg protein g^{-1} support, were 15 s and 33 s, respectively. Mycobacterium E3 organisms immobilized on lava lost half of their activity in 250 h. while organisms immobilized on perlite lost half of their activity in 60 h. Although the operational stability of the biocatalyst should be improved and the biocatalyst is only suitable at temperatures above 10°C, the application of ethene-utilizing bacteria in a gas/solid bioreactor appears attractive.

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INTRODUCTION

Ethene is the only gaseous plant hormone which physiologically is still active at very low concentrations. However, it may also cause the deterioration of harvested fruits, vegetables and flowers (Abeles, 1973; Beyer, 1981; Jaarverslag Sprenger Instituut, 1983). The threshold concentration for flowers like carnations (Jaarverslag Sprenger Instituut, 1983) is for instance 0.06 vpm. Ethene is produced by several sources like ripening fruit , exhaust gases from traffic and emissions from industries. Consequently ethene can accumulate in warehouses in which fruits, flowers and vegetables are stored. A continuous removal of ethene to very low concentrations from such places is therefore desirable. The most widespread solution for the removal of ethene is ventilation. However, ventilation is impractical when the temperature and/or the gas composition in the warehouse is different from the open air or when the latter contains too much ethene. Some chemical and physical procedures for the removal of ethene are known and the use of potassium permanganate and UV light seem economically viable methods not subject to some of the drawbacks of ventilation (Wills et al., 1983; Rudolphij and Boerrigter; 1981). In our laboratories we are working with alkene-utilizing bacteria (de Bont, 1976; de Bont and Harder, 1978; Habets-Crützen et al., 1984) which produce 1,2-epoxyalkanes in, for instance, a gas/solid bioreactor (de Bont et al, 1983). We have also attempted to use these bacteria in the same bioreactor to scrub ethene from the air in storage facilities. In this paper we report on the results of experiments made to study the performance of immobilized ethene-utilizing bacteria at very low ethene concentrations. This study is important when considering the utility of a bioscrubber to remove ethene from warehouses.

MATERIALS AND METHODS

Material. All chemicals were obtained from Merck of Darmstadt and the gases from AGA, Amsterdam. The lava was a gift of Gebr. Rook, Krimpen a/d IJssel and perlite was obtained from Pull B.V., Rhenen. The lava and perlite used had densities of 1.9 kg m^{-3} and 0.09 kg m^{-3}, respectively. The average size of both carriers was 2-2.4 mm. Alginate beads had a diameter of 1.2 mm.
Micro-organisms. The isolation and description of Mycobacterium E3 used in this study has been reported by Habets Crützen et al. (1984).
Cultivation of the organism. Organisms were grown in a 3 dm^3 fermenter. Ethene and air were supplied by two mass-flow controllers at a rate of 1.5 and 60 cm^3 min^{-1}, respectively. The mineral medium used has been described

by Wiegant and de Bont (1980) The pH was maintained at 7, and the temperature at 30°C.

Immobilization procedures. Mycobacterium E3 organisms were immobilized in alginate (as described by Habets-Crützen et al. (1984)) on lava or perlite by adding the Mycobacterium E3 organisms in a 50 mM phosphate buffer (pH=7) to the carrier. One cm^3 of the bacterial suspension was added either to lava (6.9 g) or to perlite (0.5 g).

Analysis. Gas chromatographic determination of ethene has been described previously (de Bont et al., 1979) except that the samples were 0.5 cm^3 instead of 0.1 cm^3. The protein concentration was determined as described by Habets-Crützen et al. (1984).

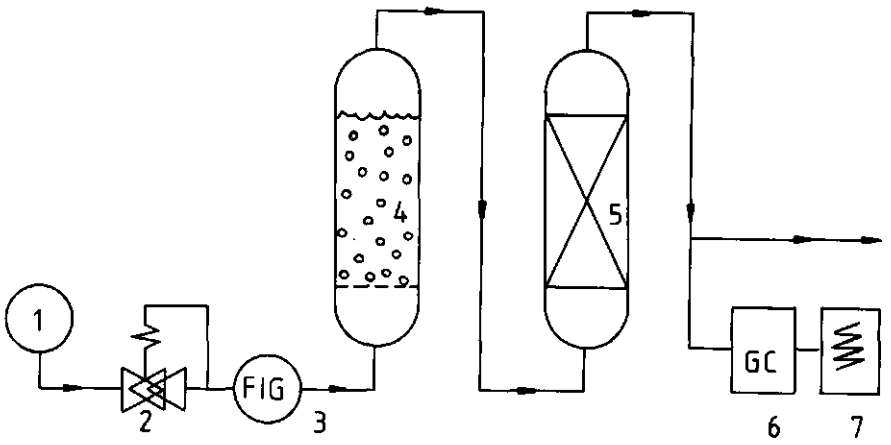

Figure 1. Schematic representation of a gas/solid bioreactor. The bioreactor consists of a gas supply system with a gas container with 3.2 vpm ethene in air mixture (1); a pressure regulator (2) and a mass-flow controller (3). The gas flow is passed through a humidifier (4) and a 5.5 cm^3 packed bed (5), respectively. Ethene concentrations in the outgoing gas were automatically measured by a gaschromatograph (6) coupled to an integrator (7). The temperature of the bioreactor may be controlled by a thermostat.

Activity assays. The rate of ethene consumption by free or immobilized mycobacteria was measured by incubating the cell suspensions in a phosphate buffer (50 mmol dm^{-3}; pH=7.2) in 17 cm^3 screw-cap bottles which were then shaken vigorously at 30°C. Reactions were started by injecting the appropriate amount of ethene through the butyl septa of the screw-cap bottles. In experiments to determine the maximal activities the initial concentration of ethene in the gas phase was 3000 vpm. Samples were withdrawn from the gas phase at regular intervals for gas chromatographic analysis. Maximal activities were calculated from the slopes of concentration vs time

curves.

To determine Michaelis Menten constants for free and immobilized organisms the initial concentrations of ethene used were 300 vpm and for cells immobilized in alginate the initial concentration was 1000 vpm. Samples from the gas phase were withdrawn at regular intervals for gas chromatographic analysis. Michaelis Menten constants were calculated by fitting the integrated Michaelis Menten equation to the measured concentration-time data by means of a computer program.

Stability tests. Operational stability was tested in a gas/solid biorector (Figure 1) operated continuously. The gasflow of the mixture of 3.2 vpm ethene in air was 2.5 cm^3 min^{-1} and the temperature was maintained at 30°C. Porosity of the packed bed was 0.4 for both carriers. The storage stability of free organisms was determined by measuring the activity at regular intervals at 30°C. The Mycobacterium E3 organisms were stored in 50 mM phosphate buffer (pH=7) at 4 and -20°C.

Degree of conversion versus residence time. This was determined in a gas/solid bioreactor with the mixture of 3.2 vpm ethene in air. The temperature was maintained at 30°C.

RESULTS AND DISCUSSION

Free organisms

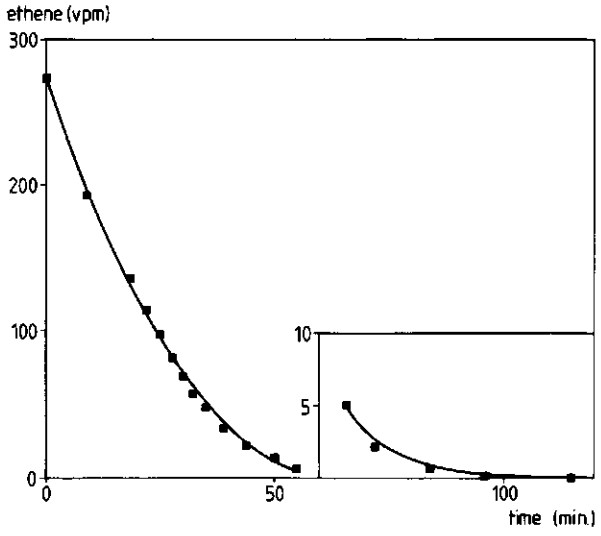

Figure 2. Oxidation of ethene to low concentrations by a washed cell suspension of Mycobacterium E3 (0.75 mg protein) at 30°C and pH 7.0. Incubations were carried out as described in materials and methods.

A prerequisite for the successful application of ethene-utilizing bacteria is the ability of the micro-organisms to oxidize ethene to concentrations low enough for the gas to be no longer physiologically active on fruits, flowers and vegetables. Therefore, an initial batch experiment using free cells of Mycobacterium E3 was carried out to determine if the organisms were in fact able to reduce the concentration of ethene to below 0.1 vpm. From Figure 2 it is clear that the bacteria indeed are able to lower the ethene concentration to the desired concentration. From these data, the K_m and V_m for ethene were calculated to be 100 vpm and 50 nmol min-1 (mg protein)$^{-1}$, respectively. Mycobacterium E3 organisms were also able to oxidize ethene at the same rate in an atmosphere of reduced oxygen levels (1%) and increased carbon dioxide levels (5%).

Apparent kinetic constants.

Apparent kinetic constants were determined for Mycobacterium E3 immobilized in various ways. Table 1 shows that about 20% of the maximal activity is lost due to immobilization in alginate and, more importantly, that the apparent K_m increases rapidly when the cell load is increased. Because of this increase in

Table 1. Apparent kinetic constants for ethene of Mycobacterium E3 immobilized on lava (density; 1.9 kg m-3), perlite (density 0.09 kg m-3) or in alginate.

Support matrix	Cell load mg protein g^{-1} dry support matrix	K_m vpm	V_m nmol min^{-1} mg^{-1} of protein
Lava	0.72	103	45
Lava	0.36	93	50
Lava	0.18	96	48
Lava	0.09	75	47
Perlite	18.5	142	43
Perlite	11.0	90	49
Perlite	5.5	107	48
Perlite	2.8	107	50
Alginate	150.0	710	41
Alginate	70.0	690	39
Alginate	20.0	470	38

apparent K_m this immobilization method was not further investigated since in a bioscrubber the biocatalyst is required to

oxidize ethene at the highest possible rates, even at very low concentrations.

Immobilization of bacteria on lava and perlite did not significantly alter the apparent kinetic constants as compared to free organisms. Furthermore, no significant effect of cell loading of the supports on these parameters was observed indicating that diffusion limitation played no role encouraging the use of the immobilization processes employed.

Operational stability.

The operational stability of Mycobacterium E3 immobilized on lava or perlite will also be of critical importance in operating a bacterium-based ethene scrubber. Operational stability was tested by placing the immobilized cells in a continuous gas stream of 3.2 vpm ethene in air and by periodically monitoring the ethene concentration in the gas stream leaving the system (Figure 1). Mycobacterium E3 immobilized on perlite reduced the ethene concentration to below 1 vpm for 25 h while Mycobac-

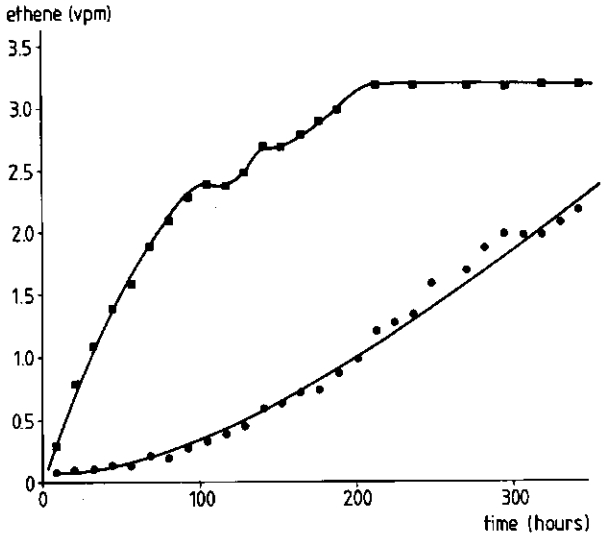

Figure 3. Operational stability at 30°C of Mycobacterium E3 immobilized on lava (●) and on perlite (■) as shown by the ethene concentration in the outcoming gas. Cell loads were 0.36 mg protein g^{-1} lava and 5.5 mg protein g^{-1} perlite. The flow of the incoming gas (a 3.2 vpm ethene in air mixture) was 2.5 cm^3 min^{-1}.

terium E3 immobilized on lava kept the ethene concentration in the outgoing gas below 1 vpm for 200 h (Figure 3). At present

it is not clear why the operational stability of Mycobacterium E3 on lava is much better than on perlite and based on these results, only lava was used in further experiments. However, the operational stability of cells on lava is less than satisfactory and further work will concentrate on improving the stability.

Storage stability.

The immobilization of bacteria on lava is relatively easy and therefore only the storage stability of free cells was determined. Mycobacterium E3 did not lose activity when stored for more than two months at -20°C, whereas when stored at 4°C these organisms lost half of their activity within two days.

Temperature

Temperature is an important parameter for the immobilized biocatalyst, because fruits, flowers and vegetables are stored at temperatures ranging from -2 to 20°C. Figure 4 shows an Arrhenius plot for both free and immobilized cells. The activation energy of the reaction is 7.1 kJ mol-1 ; the biocatalyst

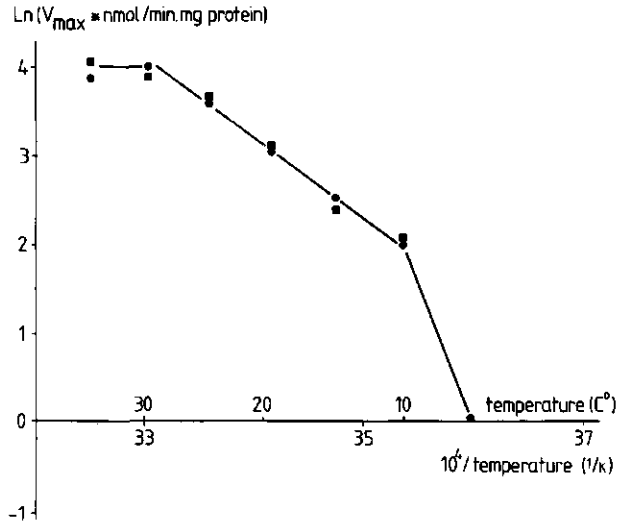

Figure 4. Arrhenius plot of free (■) and on lava immobilized (●) cells of Mycobacterium E3 at pH 7.0. The cell load of the biocatalyst was 0.72 mg protein g-1 lava.

is almost inactive at 4°C. The possible application of Mycobacterium E3 thus seems to be limited to temperatures between 10 and 35°C. To reduce the energy costs for bioscrubbing while

storing at -2 to 10°C, either an organism with considerable higher maximal activities should be obtained or a psychrophilic ethene-utilizing bacterium should be isolated.

Degree of conversion versus residence time

The effect of the residence time of the ethene containing gas mixture in the bioscrubber on the degree of conversion of ethene is shown in Figure 5. The effect of cell load on the ethene removal as effected by the residence time was also investigated. The biocatalyst with the highest cell load (0.72 mg protein g^{-1} lava) reduced the ethene concentration to below 1 vpm at a residence time of 15 s while at a lower cell load (0.36 mg protein g^{-1} lava) a residence time of 33 s was required to reach the 1 vpm level.

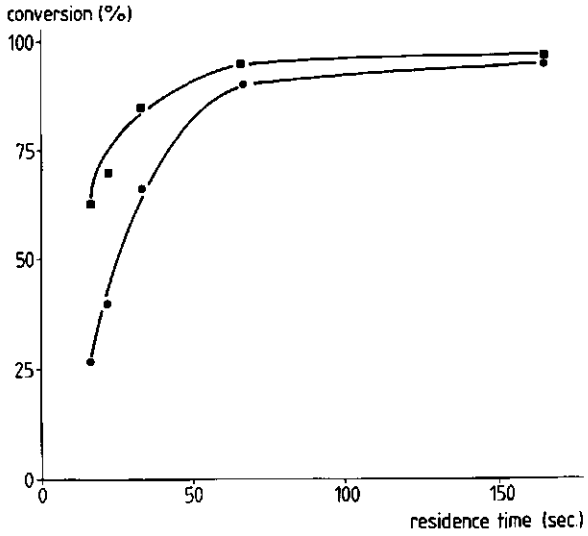

Figure 5. Effect of residence time on conversion of ethene in a gas/solid bioreactor using Mycobacterium E3 immobilized on lava at 30°C. The cell load was 0.72 mg protein g^{-1} lava (■) and 0.36 mg protein g^{-1} lava (●) and the ethene concentration in the incoming gas was 3.2 vpm.

CONCLUSION

Preliminary results encourage the view that immobilized Mycobacterium E3 may be used in an effective bioscrubber for ethene since they are able to oxidize ethene to below 0.1 vpm and because the cells do not lose actvity upon immobilization on

carriers like lava or perlite. Mycobacterium E3 immobilized on lava reduced the ethene concentration of a 3.2 vpm ethene in air mixture to below 1 vpm for 200 h. Nevertheless a better operational stability is a prerequisite for the development of a practical bioscrubber for ethene removal. From the efficiency of conversion of ethene and the rate of ethene production by fruits, vegetables and flowers, it can be calculated that the bioscrubber could be of relatively small dimensions in relation to the storage facilities.

Acknowledgments; We are indebted to Dr Ir J. Tramper, Prof Dr W. Harder and Prof Dr Ir C.J.E.A. Bulder for advice in preparing the manuscript. These investigations were supported (in part) by the Programma Commissie Biotechnologie.

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CHAPTER 10

GROWTH AND STABILITY OF ETHENE-UTILIZING BACTERIA ON COMPOST AT VERY LOW SUBSTRATE CONCENTRATIONS

C.G. van Ginkel, H.G.J. Welten and J.A.M. de Bont

SUMMARY

Ethene-utilizing micro-organisms on compost may be applied in a packed bed to scrub the plant hormone ethene from air. Ethene at the concentrations tested (50 - 200 vpm) supported growth of micro-organisms present in compost and also of ethene-grown Mycobacterium E3 cells immobilized on compost.

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INTRODUCTION

Ethene is a gaseous plant hormone that has profound, sometimes spectacular effects on physiological processes at very low concentrations. Not only desirable effects are caused by ethene but it also brings about the deterioration of stored fruit, vegetables and flowers (1). Ethene is produced by stored fruits and vegetables and in some cases it is impossible to lower the ethene concentrations in warehouses by ventilation. We therefore are trying to develop a gas/solid bioreactor with ethene-utilizing micro-organisms to scrub ethene at very low concentrations (0.1 - 2.0 vpm) from these warehouses (2).

Such ethene-utilizing organisms thus should be able to oxidize and possibly grow on ethene at these very low concentrations. It may be anticipated that such organisms are present in soil since traces of ethene are produced in soils by plants and micro-organisms (3,4) and since the gas does not accumulate in soil under aerobic conditions. It indeed has been demonstrated that the breakdown of ethene to very low concentrations in soil was mediated by aerobic bacteria (5,6,7,8) and pure cultures of ethene-utilizing bacteria were isolated (7).

At present it is not clear whether at ethene concentrations in the 0.1 - 2.0 vpm range the organisms would also be able to use energy from the ethene oxidized at these low concentrations, either for maintenance purposes to sustain the population and its oxidative capacity, or even to grow. The ability of the organisms to at least sustain the oxidative capacity for a prolonged period of time is of great importance when considering the application of the cells to continuously scrub ethene at low concentrations from atmospheres. In the previous paper (2) it was observed that loss of cell activity occurred at low ethene concentrations within a few days when the cells were immobilized on lava or perlite. The ethene-utilizing bacteria have been immobilized on compost and in this paper we describe the behaviour of the immobilized cells at very low ethene concentrations.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Janssen Chimica, Beerse, Belgium and the gases were obtained from AGA, Amsterdam, The Netherlands. Compost was obtained from V.A.M., Amsterdam, The Netherlands. The compost was dried overnight at a temperature of 150°C when it was used as a carrier for Mycobacterium E3. Perlite was obtained from Pull BV Rhenen, The Netherlands.

Micro-organisms. The isolation and characterization of Mycobacterium E3 has been described previously by Habets-Crützen et al. (9).

Cultivation of the micro-organisms. Mycobacterium E3 was cultivated in mineral salts medium described by Wiegant and de Bont (10) using a 3 dm^3 fermentor. Ethene and air were supplied by two mass flow controllers at a rate of 2.5 and 100 cm^3/min, respectively. Cultivation temperature was 30 °C and the pH of the culture was 7.0.

Preparation of washed cell suspensions. Preparation of washed cell suspensions has been described previously by de Bont et al. (11).

Analysis. The protein concentration was determined as described by Habets-Crützen et al. (9).

Immobilization on compost. Mycobacterium E3 was immobilized on compost by slowly adding 1 cm^3 of a washed cell suspension to 1 gr of dry compost while mixing the compost with a spatula.

Gas/solid bioreactor. The gas/solid bioreactor consists of a gas supply system with gas containers with 2.0, 50 or 200 vpm ethene in air, a pressure regulator and a mass flow controller. The gasflow is passed through a humidifier with demineralized water and a packed bed. The 15 cm^3 packed bed had a length of 10 cm. The porosity of the bed was 0.2 and the temperature of the packed bed was maintained at 30°C. Ethene concentrations of the outflowing gas were assayed automatically by flame ionization gas chromatography with a porapak R column (11).

Oxidation of ethene by immobilized micro-organisms. The oxidation of ethene by ethene-utilizing micro-organisms was determined in the gas/solid bioreactor. Ethene in air was supplied at various concentrations and flow rates, and the operational stability and possible growth of the micro-organisms was studied by measuring the ethene content of the outflowing gas.

Uptake of ethene by compost. 10 g Compost was incubated at 30 ° C in 100 cm^3 screw-cap bottles and 0.1 cm^3 ethene was injected into the bottle. Ethene oxidation was measured by gas chromatography as described by de Bont et al. (11) and oxygen concentrations were checked regularly to assure aerobic conditions.

RESULTS AND DISCUSSION

Growth of ethene-utilizing micro-organisms on compost

When soils were sterilized no oxidation of ethene occurred showing that micro-organisms are responsible for the disappearance of ethene in soil (5,6,8). Ethene-utilizing bacteria are also present in compost because 1000 vpm ethene disappeared within 4 days from the gas phase of a closed bottle containing 10 g compost. To study whether these micro-organisms were also able to grow at low concentrations of ethene, compost was placed in the gas/solid bioreactor and ethene in air mixtures of 2 vpm, 50 vpm or 200 vpm, respectively were passed through

the packed bed to initiate growth of ethene-utilizing micro-organisms present on compost. With the 2 vpm ethene in air mixture a change in the ethene concentration in the outflowing gas was detected after one month suggesting that even at such low concentrations, growth of micro-organisms already present on compost might be possible or enzymes responsible for the oxygenation of ethene were induced (Fig. 1). With the 50 vpm and 200 vpm ethene in air mixtures a decrease of the ethene concentration in the outflowing gas was already detected after one to two weeks indicating growth of the organisms (Fig 1).

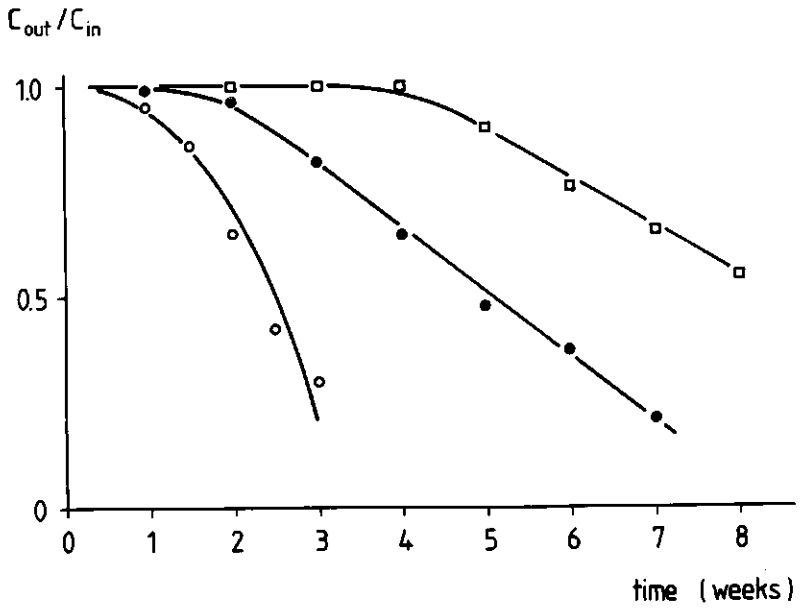

Figure 1. Enrichment of ethene-utilizing micro-organisms on 4 g compost in a gas/solid bioreator with 2.0 vpm (□), 50 vpm (●) and 200 vpm (○) ethene in air mixtures at a flow rate of 1.3 cm^3/min as shown by the conversion.

These results are consistent with results obtained by several authors who studied the oxidation of ethene in soils in batch systems (6,7,8). For instance, with clay and sandy soils the rate of ethene uptake accelerated after approximately one week of incubation if the ethene concentration was higher than 20 vpm, also indicating growth (7). Direct evidence that micro-organisms are involved has been provided by the isolation of ethene-utilizing Mycobacterium from these soils (7). From the compost used in the gas/solid bioreactor we have also isolated

ethene-utilizing bacteria and from microscopic observations, it was obvious that they strongly resembled the previously isolated mycobacteria. Certain Xanthobacter spp. and Nocardia spp. are also known to utilize ethene (12) but such strains were not isolated from the compost.

Growth of Mycobacterium E3 on compost at low concentrations

Ethene concentrations in warehouses must be 2 vpm or lower to be no longer physiologycally active in fruits and vegetables. But with 2 vpm ethene in the air it took more than one month before a good ethene-removing system was established and it can therefore be concluded that the compost should be inoculated with ethene-grown bacteria prior to use in a bioscrubber of warehouses of fruits and vegetables. Compost for that reason was inoculated with ethene-grown Mycobacterium E3 and was given

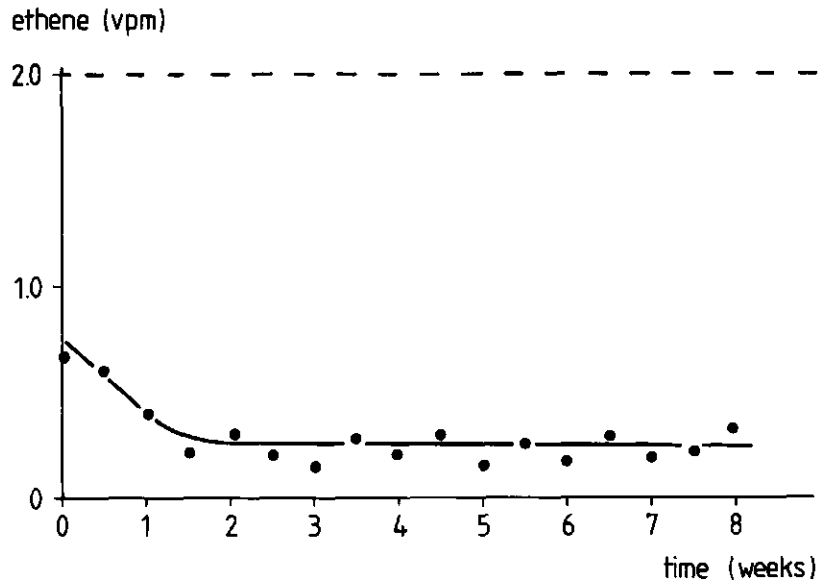

Figure 2. Operational stability of Mycobacterium E3 (15 mg protein) immobilized on 4 g compost in a gas/solid bioreactor at 2 vpm ethene in air at a flow rate of 8 cm^3/min. Closed dots represent the ethene concentration of the outflowing gas.

a 2 vpm ethene in air mixture. Removal of ethene from the gas phase was immediate and a slight improvement in the conversion of ethene during the first two weeks was detected which might be due to possible growth on ethene or to mixotrophic growth on ethene and compost compounds (Fig. 2). Degradable compounds

like cellulose, lignine and protein could be responsible for the good operational stability of Mycobacterium E3 on compost and/or possible mixotrophic growth. Therefore, these carbon and energy sources were added to the inert carrier perlite but these additions did not result in a better operational stability indicating that mixotrophic growth is probably not responsible. The operational stability of Mycobacterium E3 on compost was also tested with various cell loads and no differences in stability were recorded pointing out that the stability was not caused by an excess of cells on the carrier either. In a subsequent experiment it was further demonstrated that Mycobacterium E3 immobilized on compost remained viable when starved from ethene for two weeks.

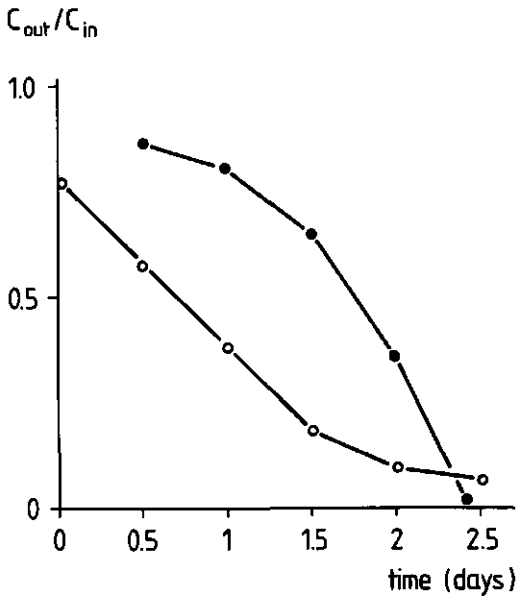

Figure 3. Growth of Mycobacterium E3 (2 mg protein) immobilized on 4 g compost in a gas/solid bioreactor with a 50 vpm (○) and 200 vpm (●) ethene in air mixtures at a flow rate of 1.3 cm^3/min as shown by the conversion.

Another system for the removal of traces of gases by a pure culture was investigated by Brisbane and Ladd (13) who studied the oxidation of ethane at very low concentrations in soil with added Mycobacterium paraffinicum. When soils were incubated with 5 vpm of ethane in air there was after three months no increase in activity as compared to soils that were incubated under air suggesting that Mycobacterium paraffinicum did not grow at these low concentrations. Mycobacterium paraffinicum

immobilized on soil remained viable over twelve weeks of incubation with 5 vpm ethane and even with only air. This agrees with the results obtained with Mycobacterium E3 on compost.

Growth at 50 vpm and 200 vpm ethene in air of Mycobacterium E3 immobilized on compost was obvious because the ethene concentration in the outflowing gas was reduced to 5 vpm in only 3 days (Fig. 3). Differences in the curves between the 50 vpm and 200 vpm ethene are probably caused by a higher growth rate of Mycobacterium E3 at 200 vpm ethene than at 50 vpm ethene. The Michaelis Menten constant of Mycobacterium E3 for ethene is approximately 100 vpm (12). Ethane-utilizing Mycobacterium paraffinicum added to soils can grow at ethane concentrations of 15 vpm or higher (13) while Rusakova (14) demonstrated growth of Mycobacterium perrugosum at 28 vpm ethane in air. However, when comparing the lower limits of concentrations at which growth of cells on gaseous compounds occurs, it should be realized that the solubility of these gases in water varies greatly.

Compost is used in biofilters and these bioreactors in general have at least two disadvantages namely a poor mass transfer and a high pressure drop which are due to the nature of the support material. These latter disadvantages would demand for a better and more defined support than compost but unfortunately the stability of cells on such supports is poor (2). It thus seems very desirable to arrive at a system that would combine the operational stability of cells on compost with the better process characteristics of materials as for instance lava or perlite. To this we in the future will study the fundamental aspects of the physiology of the ethene-utilizing bacteria in connection with the stability on various support materials.

Acknowledgements; We are grateful to Prof. Dr. W. Harder and Prof. Dr. Ir. C.J.E.A. Bulder for discussions. These investigations were supported by the Programma Commissie Biotechnologie.

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CHAPTER 11

SAMENVATTING

Gasvormige alkenen komen in het milieu door emissie van de industrie en door de uitscheiding van o.a. etheen door planten, schimmels en bacteriën. Zoals te verwachten is, hebben een aantal microörganismen de mogelijkheid verworven om deze verbindingen te oxideren. Deze oxidatie wordt vooral uitgevoerd door alkaangebruikende bacteriën die alkenen co-oxideren en door alkeengebruikende bacteriën die onverzadigde koolwaterstoffen als koolstof- en energiebron gebruiken. De alkaangebruikende bacteriën zijn reeds uitgebreid onderzocht en beschreven, en potentiële toepassingen van deze microörganismen zoals de epoxidatie van alkenen zijn in patenten vastgelegd.

Dit onderzoek is een onderdeel van interdisciplinair microbiologisch/proceskundig werk dat gericht was op het verwerven van fundamentele kennis over alkeengebruikende bacteriën in tweede generatie bioreactoren zoals de gas/vast bioreactor en de multifase bioreactor. In dit proefschrift komen voornamelijk microbiologische aspecten aan de orde.

De hoofdstukken 2, 3 en 4 gaan over respectievelijk de isolatie en karakterisering van Xanthobacter spp. die op propeen en 1-buteen kunnen groeien, Nocardia spp. die 1,3-butadiëen en isopreen als koolstof- en energiebron kunnen gebruiken, en een Nocardia sp. die op trans-2-buteen kan groeien. De initiële oxidatie van propeen, 1,3-butadiëen en isopreen tot de respectievelijke epoxides vindt plaats via een monooxygenase. Op grond van de substraat specificiteit en de activiteit in aanwezigheid van potentiele remmers bleek dat het alkeen-monooxygenase in een Xanthobacter sp. verschilt van de reeds bekende koolwaterstof-monooxygenases. In tegenstelling tot de 1-alkeengebruikende bacteriën kan de op trans-2-buteen geïsoleerde bacterie, Nocadia TB1, veel beter op gasvormige verzadigde dan onverzadigde koolwaterstoffen groeien. Uit verder onderzoek bleek dat deze bacterie een monooxygenase bezit dat trans-2-buteen via crotonyl alcohol en niet via 2,3-epoxybutaan afbreekt. De afbraakroute van trans-2-buteen in Nocardia TB1 is analoog aan die van de reeds bekende afbraakroute van butaan via boterzuur hetgeen met behulp van simultane adaptatie, meting van enzym activiteiten en remmingsproeven is aangetoond.

Bacteriën gekweekt op alkenen kunnen mogelijk toegepast worden bij de productie van (chirale) epoxyalkanen of bij de verwijdering van gasvormige alkenen uit gasfases. Dit maakt een optimale productie van deze bacteriën noodzakelijk. Groei van een tweetal alkeengebruikende bacteriën in chemostaat culturen

op de gasvormige verbindingen is in hoofdstuk 5 behandeld. Met behulp van een eenvoudig model en experimenteel verkregen groeiparameters van een Xanthobacter sp. en een Mycobacterium sp. kon de verdunningssnelheid berekend worden waarbij de biomassa productie optimaal is. Deze berekende waarden bleken goed met de experimenteel bepaalde productiviteit overeen te komen.

Alkeengekweekte rustende cellen van Xanthobacter spp. hebben de potentie om epoxyalkanen uit alkenen te vormen zoals uitgebreid beschreven is in hoofdstuk 6. Deze vorming van epoxiden is een gevolg van de brede substraatspecificiteit van het alkeen-monooxygenase terwijl de enzymen die epoxides omzetten een minder breed spectrum van substraten aankunnen. Hoofdstuk 7 is een algemeen overzicht van wat er thans bekend is over tot nu toe geïsoleerde alkeengebruikende bacteriën. De groei van een aantal geselecteerde bacteriën op koolwaterstoffen en de oxidatie van alkenen en alkanen door alkeengekweekte bacteriën werden bestudeerd. Daarbij kwam ondermeer naar voren dat de trans-2-buteengebruikende Nocardia TB1 en de 1-hexeengebruikende Pseudomonas meer verwantschap vertonen met alkaangebruikende bacteriën. Alkeengebruikende bacteriën waren in staat een of meer epoxiden uit te scheiden en door gebruik te maken van een goede combinatie bacterie/alkeen kan bijna ieder gewenst epoxide geproduceerd worden. Tot slot wordt het lot van de gevormde epoxiden besproken waarbij naar voren komt dat de epoxiden niet alleen tot koolzuur en water geoxideerd worden maar ook worden gehydrolyseerd.

De hoofdstukken 8, 9 en 10 behandelen een aantal aspecten van het gedrag van deze bacteriën in gas/vast bioreactoren. 1,2-Epoxyethaan vorming met op propeen gekweekte Mycobacterium Py1 cellen werd in de gas/vast bioreactor bestudeerd om ophoping van het toxische product te voorkomen. Hierbij werd aangetoond dat de stabiliteit van de biokatalystor o.a. afhankelijk was van co-factor regeneratie en de 1,2-epoxyethaan productie kon dan ook verhoogd worden door gebruik te maken van een metaboliseerbaar co-substraat.

Etheen is een plantenhormoon dat bederf van agrarische producten kan veroorzaken en dient daarom verwijderd te worden uit de omgeving waar agrarische producten worden bewaard. Microorganismen, die op gasvormige alkenen kunnen groeien zijn misschien een alternatief voor de reeds bekende chemisch/fysische verwijderingsmethoden. Het is daarbij essentieel dat etheen tot zeer lage concentraties geoxideerd wordt. Op etheengekweekte Mycobacterium E3 cellen bleken na immobilisatie op lava in staat om etheen tot voldoende lage concentraties uit de lucht te verwijderen. Met behulp van conversie versus tijd proeven, en op basis van de productiesnelheid van etheen door

verschillende agrarische producten kon berekend worden dat het reactorvolume relatief klein kan zijn in relatie tot de opslagplaats van groente en fruit. De operationele stabiliteit van Mycobacterium E3 geimmobiliseerd op lava was echter onvoldoende. Gelukkig was de operationele stabiliteit van Mycobacterium E3 geimmobiliseerd op compost goed.

SUMMARY

Gaseous alkenes are widespread in the environment due to the emission of these hydrocarbons by industry and due to their production from natural sources as for instance ethene by plants, fungi and bacteria. Micro-organisms have developed the potential to oxidize these hydrocarbons. Alkenes can either be co-oxidized by alkane-utilizing bacteria and/or used as carbon and energy source by specific alkene-utilizing bacteria. Alkane-utilizing micro-organisms have extensively been investigated and described. Potential applications of alkane-utilizing micro-organisms e.g. the epoxidation of alkenes have been recorded in numerous patents. The scope of the research presented was to gain, in a concerted action between microbiologists and process engineers, more fundamental knowledge on the behaviour of alkene-utilizing bacteria in either a gas/solid bioreactor or in a multiphase bioreactor. This thesis mainly deals with microbiological aspects.

Chapters 2, 3 and 4 subsequently deal with the isolation and characterization of propene- and 1-butene-utilizing Xanthobacter spp., with Nocardia spp. utilizing both 1,3-butadiene and isoprene as sole source of carbon and energy and with a Nocardia sp. capable of growth on trans-2-butene. The initial oxidation of propene, 2-butene, 1,3-butadiene and isoprene by these bacteria is mediated by a mono-oxygenase. The mono-oxygenase present in propene-grown Xanthobacter spp. is different from hydrocarbon mono-oxygenases described until now in view of substrate specificity towards hydrocarbons and in view of activities measured in the presence of potential inhibitors of the mono-oxygenase. Both Xanthobacter spp. and alkadiene-utilizing Nocardia spp. possess a mono-oxygenase which catalyses an epoxidation reaction. On the other hand, the trans-2-butene-grown Nocardia sp. which is also able to grow on gaseous n-alkanes, carries out a hydroxylation reaction instead of an epoxidation reaction. A degradation route of trans-2-butene via crotonic acid was proposed on basis of inhibitor experiments, simultaneous adaptation studies and enzyme activities.

Possible applications of alkene-grown bacteria are the production of (chiral) epoxyalkanes and the removal of alkenes from gas phases and therefore an optimal production of the bacteria is essential. Microbial growth on either ethene or propene in chemostat cultures is dealt with in chapter 5. By using a simple growth model and experimentally derived growth parameters with a Xanthobacter sp. and a Mycobacterium sp. the dilution rate resulting in the optimal biomass production could be calculated. Measured and mathematically derived production rates agreed well.

The potential to produce epoxyalkanes from alkenes was investigated using washed cell suspensions of alkene-grown Xanthobacter spp. The results are given in chapter 6. Chapter 7 represents a extended survey of gaseous hydrocarbon utilization and oxidation by alkene-grown bacteria. Some selected alkene-utilizing micro-organisms were investigated in more detail to provide a better understanding of the ability to grow on hydrocarbons and the oxidation of gaseous and volatile hydrocarbons. From such observations it was obvious that a 1-hexene-utilizing Pseudomonas and a trans-2-butene-utilizing Nocardia TB1 resemble alkane-utilizing bacteria. Other alkene-utilizing micro-organisms consist of a specific group, which are not able to hydroxylate alkanes. Alkene-grown bacteria were capable to excrete epoxides and using an appropriate combination of bacterium and alkene almost every epoxide could be produced. Finally, it was shown that most epoxides formed were either oxidized to CO_2 and H_2O, or hydrolysed by alkene-grown bacteria.

Chapter 8, 9 and 10 deal with some aspects of the behaviour of alkene-utilizing bacteria in gas/solid bioreactors. 1,2-Epoxyethane formation by propene-grown Mycobacterium Py1 cells was studied in such a reactor because no accumulation of the toxic epoxide occurs in the vicinity of the bacteria. Prolonged 1,2-epoxyethane formation was dependent on co-factor regeneration. In a subsequent experiment, it was demonstrated that the presence of a metabolizable co-substrate enhanced the epoxide production.

Ethene is a plant hormone and has already detrimental effects on stored fruits and vegetables at concentrations of 1 vpm in the gas phase. Therefore, ethene has to be removed from the vicinity of stored agricultural products. Alkene-grown bacteria capable of oxidizing ethene may be an alternative of known chemical/physical ethene-removal systems from storage facilities. Ethene-grown Mycobacterium E3 oxidizes ethene to the desired low concentrations even when immobilized on carriers like lava, perlite or in alginate. Chapter 9 describes the characteristics of ethene-grown Mycobacterium E3 immobilized on various supports. However, the operational stability of Mycobacterium E3 immobilized on the supports tested was insufficient. In a subsequent investigation the operational stability of Mycobacterium E3 on compost was tested, and surprisingly, a good operational stability was found while possibly even cell-growth or induction of mono-oxygenase enzyme was obtained also at very low ethene concentrations. From the efficiency of conversion of ethene and the rate of ethene production by fruits and vegetables, it was calculated that bioscrubbers can be of relatively small dimensions in relation to storage facilities.

CURRICULUM VITAE

De auteur van dit proefschrift werd te Bunnik geboren op 2 december 1955. Na het Chr. Lyceum te Zeist doorlopen te hebben werd in 1975 begonnen met de studie Milieuhygiëne aan de Landbouwhogeschool te Wageningen. Deze studie werd in 1982 afgesloten. In 1982 werd gestart met een promotieonderzoek bij de Vakgroep microbiologie van de Landbouwhogeschool.