BIOCATALYSTS FOR PRODUCTION OF CHIRAL EPOXIDES

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Abstract

An extensive screen for microorganisms capable of growth on olefins was carried out. Over 100 bacterial strains (mesophilic, thermophilic) were isolated in pure culture with gaseous or liquid (internal & terminal) olefins as the sole source of carbon and energy. The organisms included *Aerococcus*, *Aeromonas*, *Alcaligenes*, *Flavobacterium*, *Micrococcus*, *Moraxella*, *Nocardia*, *Pseudomonas*, *Staphylococcus*, *Streptomyces* and *Vibrio* spp. and a variety of Gram-negative, Gram-positive and Gram-variable rods/coccobacilli not yet identified. Given the success of isolating many different genera of alkene-utilizing bacteria, it would suggest that the ability to utilize olefin hydrocarbons is more widespread in nature than previously recognized.

Initial studies involved testing a collection of strains for i) growth rate on the substrate of isolation and ii) evidence of epoxide accumulation from lower gaseous olefins. All of the 18 ethene- and propene-utilizing bacteria tested, stereospecifically formed *R*-1,2-epoxypropane (ee=90-96%), *R*-1,2-epoxybutane (ee=90-98%) and *trans*-(2*R*,3*R*)-epoxybutane (ee=64-88%), as analyzed by chiral complexation glc. A particularly promising strain of *Micrococcus* sp. M90C was selected to demonstrate:

- a) Induction of epoxidation activity in carbohydrate-grown cells.
- b) Optimization of a biotransformation process for production of epoxyethane.
- c) Involvement of a specific monooxygenase in the formation of chiral epoxides.
- d) Stereoselectivity of epoxyalkane degradation.
- e) Substrate specificity toward a range of olefins.

Micrococcus sp. M90C stereospecifically formed *S*-(+)-phenyl glycidyl ether (ee=93%) and *R*-(+)-phenyl methyl sulphoxide (ee>98%), as analyzed by chiral-shift NMR and HPLC. The ability to catalyze heteroatom oxygenation as well as epoxidation of a wide range of olefins in high optical and chemical yields, demonstrates its potential as a 'general-purpose' biocatalyst for the production of optically pure oxygenated compounds.

Two other promising ethene-utilizing bacteria (M26, M93A) each catalyzed the stereospecific formation of *S*-(+)-phenyl glycidyl ether (ee=93%). This has attracted considerable industrial interest and is currently under investigation at ICI for commercial production of optically active β -blockers. In addition to *Micrococcus* sp. M90C, the substrate specificities of 6 other organisms were studied in order to probe the active site of the enzymes involved. The pattern of reactivity for the group of 4 ethene- (M26, M90C, M93A, M186) and 2 propene- (M142, M156) utilizers differed from that expected with peracids, suggesting a considerable degree of enzymatic control. The nature of the substrate, however, modulated enzymatic epoxidations in *Staphylococcus* sp. M97B.

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Abbreviations

The following abbreviations are used in the text:

ATCC	American Type Culture Collection
BME	2- β -mercaptoethanol
BSA	bovine serum albumin
CoA	coenzyme A
CSR	chiral-shift reagent
DNA	deoxyribonucleic acid
DW	dry weight
EDTA	ethylenediaminetetraacetic acid
ee	enantiomeric excess
FID	flame ionization detector
GC	gas chromatograph
glc	gas liquid chromatography
HPLC	high performance liquid chromatography
id	internal diameter
IFO	Institute for Fermentation Osaka
m/e	mass to charge ratio
MS	mass spectrometer
NAD ⁺	β -nicotinamide adenine dinucleotide, oxidized form
NADH	β -nicotinamide adenine dinucleotide, reduced form
NAD(P)H	β -nicotinamide adenine dinucleotide phosphate, reduced form
NCIB	National Collection of Industrial Bacteria
NCIMB	National Collection of Industrial & Marine Bacteria
NMR	nuclear magnetic resonance
ppm	parts per million
psi	pounds per square inch
r	radius
RNA	ribonucleic acid
rpm	revolutions per minute
Tris	tris(hydroxymethyl)aminomethane

CHAPTER 1

INTRODUCTION

Hydrocarbon-utilizing bacteria are widely distributed in nature (Levi *et al.*, 1979). Chemical analyses have revealed the presence of significant quantities of aliphatic and aromatic hydrocarbons in soil and sediments as well as petroleum polluted areas. The probable origin of these compounds is a result of ongoing biosynthesis by certain plants, microorganisms and by the physicochemical action of decay of organic matter. The occurrence of hydrocarbon-utilizing bacteria depends on the environmental conditions. These include physical constraints such as temperature, availability of oxygen, pH and the extent to which the particular habitat is an open or closed ecosystem. Nutritional factors include the availability of utilizable sources of nitrogen, phosphorus and other elements, the nature of hydrocarbon substrate and its effective concentration, and the possible presence of toxic substances in the environment (Rosenberg & Gutnick, 1981).

The chemical heterogeneity and water insolubility of hydrocarbons pose special problems for microorganisms using them as a source of carbon and energy. The availability of hydrocarbons as growth substrates, depends on the solubility of the particular hydrocarbon in water, its physical state (solid, liquid, gas) and toxicity, which will dictate its addition as an emulsion, vapour or with an inert carrier.

McAuliffe (1966) measured the solubility of various gaseous and liquid hydrocarbons in water at room temperature and showed that olefins had considerably higher solubility than paraffins. Ring closure increased water solubility. The addition of unsaturated bonds in the ring progressively increased water solubility through the sequence cycloolefin, cyclodiolefin and aromatic hydrocarbons. Branching in general, decreased water solubility of hydrocarbons (tables 1 & 2).

Table 1. Solubility of paraffins and olefins in water at room temperature.

Paraffins	Solubility (mM)	Olefins & epoxides	Solubility (mM)
Methane	1.5	Ethene	4.7
Ethane	2.0	Propene	4.8
Propane	1.4	1-Butene	4.0
<i>n</i> -Butane	1.1	2-Methyl propene	4.7
<i>iso</i> -Butane	0.8	1-Pentene	2.0
<i>n</i> -Pentane	0.5	2-Pentene	3.0
<i>iso</i> -Pentane	0.7	3-Methyl-1-butene	1.5
<i>n</i> -Hexane	0.1	1-Hexene	0.6
<i>n</i> -Heptane	0.03	2-Heptene	0.2
<i>n</i> -Octane	0.01	1-Octene	0.02
2,2-Dimethyl propane	0.5	1,3-Butadiene	13.6
2,2-Dimethyl butane	0.2	Epoxyethane	8690.0
2-Methyl pentane	0.2	1,2-Epoxypropane	6970.0
2,4-Dimethyl pentane	0.04	1,2-Epoxybutane	819.0
2,2,4-Trimethyl pentane	0.02		

Adapted from McAuliffe, 1966

Table 2. Solubility of cyclic and aromatic hydrocarbons in water at room temperature.

Cyclic & aromatic hydrocarbons	Solubility (mM)
Cyclopentane	2.2
Cyclohexane	0.7
Cycloheptane	0.3
Cyclooctane	0.1
Methyl cyclopentane	0.5
Methyl cyclohexane	0.1
Cyclopentene	8.0
Cyclohexene	2.6
Cycloheptene	0.7
1-Methyl cyclohexene	0.5
1,4-Cyclohexadiene	8.7
Benzene	22.8
Toluene	5.6
<i>o</i> -Xylene	11.7
Ethyl benzene	1.4
Styrene	3.0
1,2,4-Trimethyl benzene	0.5
Isopropyl benzene (cumene)	0.4

Adapted from McAuliffe, 1966

1.1. Microbial epoxidation of olefins

Epoxides are useful starting materials in organic synthesis and are used as precursors in the synthesis of a variety of chemicals (Gorzynski Smith, 1984). The value of epoxides as intermediates in the total synthesis of optically active natural products or drugs emphasizes the need to obtain these compounds in a high state of enantiomeric purity. Selective direct oxygenation of organic substrates remains a largely unresolved challenge to the synthetic chemist. These reactions are difficult to control since they lack stereo- or regioselectivity and often cannot be halted at intermediate oxidation states. No general organic chemical method is yet available for the selective synthesis of enantiomeric form of epoxides, with the notable exception of asymmetric epoxidation of allylic alcohols (Katsuki & Sharpless, 1980) and the chiral formation of 1,2-epoxypropane (Golding, 1985).

Biological systems are, however, capable of stereoselective epoxidation of prochiral olefins, due to the involvement of monooxygenases. These enzymes require a reduced co-factor for catalysis, and incorporate molecular oxygen directly into organic molecules and do so with a very high efficiency and selectivity. Microorganisms attack a wide range of saturated and unsaturated hydrocarbons (Klug & Markovetz, 1971) and are able to form epoxides from alkenes, arenes and halohydrins by different enzymatic epoxidation reactions (fig. 1). Bacterial production of epoxides from aliphatic olefins has been extensively investigated during the past decade, often with the objective of exploring the possible biotechnological applications. Organisms studied include alkane-, methane- and alkene-utilizing bacteria and *Nitrosomonas europaea*. These epoxide-producing systems have often been patented (Higgins, 1980; Hou & Edison, 1982; Drozd & Bailey, 1984; Furuhashi & Takagi, 1985; Phillips *et al.*, 1988). Substrate specificity of these organisms is, however, restricted to a rather narrow range of olefins.

Figure 1. Reactions involved in microbial epoxidation.

1.1.1. Alkane-utilizers

Bacteria utilizing saturated gaseous hydrocarbons mainly belong to the genera *Brevibacterium*, *Corynebacterium*, *Mycobacterium*, *Nocardia* and *Pseudomonas* spp. (Klug & Markovetz, 1971). Most of these organisms grow well on ethane, propane or butane (Philips & Perry, 1974; Hou *et al.*, 1983; Patel *et al.*, 1983). 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 also been demonstrated (Vestal & Perry, 1969).

Most of the initial studies on microbial oxidation of olefins involved alkane-utilizing bacteria. The epoxidation of an olefin was first demonstrated by van der Linden (1963), who detected the formation of 1,2-epoxyoctane from octene by heptane-grown *Pseudomonas* cells. The epoxide was not further oxidized enzymatically. He postulated that the same enzyme system might catalyze both the hydroxylation of alkanes and the epoxidation of 1-alkenes. These early concepts were later confirmed with the *w*-hydroxylation system of *Pseudomonas oleovorans*. Octane-grown cell suspension of the bacterium catalyzes the epoxidation of terminal olefins (C_6 - C_{12}), allyl benzene and alkadienes, along with the hydroxylation of the methyl group (May & Abbott, 1972 & 1973; de Smet *et al.*, 1981b & 1983). Styrene oxide is, however, not produced (May *et al.* 1975). Metabolism of 1,2-epoxyoctane in *Pseudomonas oleovorans* occurs *via* oxidation of the methyl group and *not* by enzymatic hydrolysis and subsequent oxidation of the epoxide functionality (Abbott & Hou, 1973). Cardini & Jurtshuk (1970) found that an octane-utilizing *Corynebacterium* sp. also excretes epoxides from 1-alkenes (C_6 - C_{18}) in addition to catalyzing hydroxylation of alkanes. None of these organisms are, however, able to epoxidize gaseous (C_2 - C_4) olefins.

Hou *et al.* (1983) and Patel *et al.* (1983) reported that ethane-, propane- and butane-utilizing bacteria excrete 1,2-epoxyalkanes from gaseous olefins. The product is further metabolized enzymatically although at a very slow rate. Cell-free extracts of *Brevibacterium* sp. (strain CRL 56) are also able to epoxidize 1,3-butadiene, *cis*-/*trans*-2-butenes and even styrene in a NADH-dependent reaction. Inhibition studies suggested that an alkane

monooxygenase catalyzes both hydroxylation of alkanes and the epoxidation of olefins. 1,2-Epoxypropane is the main product of epoxidation. A trace of 3-hydroxy-1-propene is, however, detected, indicating that propene is oxidized both at the double bond and at the methyl group. This is analogous to the findings of van der Linden (1963) and May & Abbott (1973) in the oxidation of 1-octene.

Gaseous alkane-utilizing bacteria are generally not suitable as chiral epoxide producers since the epoxyalkanes are obtained in a very low optical purity (Weijers *et al.*, 1988 a). *Pseudomonas oleovorans*, however, produces the *R*-monoepoxide from 1,7-octadiene in ee=84% (May *et al.*, 1976), *R*-1,2-epoxyoctane in ee=70% (de Smet *et al.*, 1981 a) and *R*-1,2-epoxydecane in ee=60% (de Smet *et al.*, 1983). It has been shown that 1,7-octadiene is more reactive than 1-octene and 1-decene in the reactions catalyzed by the *w*-hydroxylation system (May & Abbott, 1973; May *et al.*, 1975). It is suggested that the optical purity of the enzymatic product parallels the relative reactivity of the substrates. Recently, *Pseudomonas oleovorans* has been used as a biocatalyst in the production of optically pure (ee=100%) aryl glycidyl ethers as intermediates in the synthesis of beta-blockers (Johnstone *et al.*, 1986).

1.1.2. Methanotrophs

Methane-utilizing bacteria contain a NAD(P)H-dependent monooxygenase that hydroxylates methane to methanol. In some methanotrophs, depending on the growth conditions, the methane monooxygenase (MMO) is soluble or particulate. In *Methylococcus capsulatus* (Bath), high copper concentrations stimulate formation of the particulate MMO, while copper deficiency favours formation of the soluble form of the enzyme (Stanley *et al.*, 1983). The MMO oxidizes many different substrates including alkanes, ethers, alicyclic, aromatic and heterocyclic compounds (Colby *et al.*, 1977; Higgins *et al.*, 1979). Aromatic compounds are oxidized by the soluble MMO but not by the particulate form (Burrows *et al.*, 1984). Methanotrophs also catalyze the epoxidation of gaseous internal and terminal olefins as well as styrene (Colby *et al.*, 1977; Higgins *et al.*, 1979; Hou *et al.*, 1979; Higgins

et al., 1980) but the epoxides formed are practically racemic (Subramanian, 1986; Weijers *et al.*, 1988 a). Excretion of epoxyalkanes is due to the inability to further metabolize the product (Hou *et al.*, 1979).

1.1.3. Alkene-utilizers

In nature, several gaseous and volatile olefins are produced. Ethene is a gaseous plant hormone (Abeles, 1973) produced by fungi (Lynch, 1975) and bacteria (Fujii *et al.*, 1985). The volatile isoprene and various monoterpenes are excreted by leaves of higher plants (Rasmussen, 1970). Many unsaturated hydrocarbons, especially the lower gaseous olefins (ethene, propene, vinyl chloride, 1,3-butadiene, butenes) are produced chemically on a large scale and inevitably are partly released into the environment. It is, therefore, not surprising that many aerobic microorganisms have been isolated that are able to use these compounds as carbon and energy sources.

Bacterial genera isolated on the lower gaseous olefins to date include: *Mycobacterium* spp. (de Bont, 1976; de Bont *et al.*, 1980; Drozd & Bailey, 1984; Habets-Crützen *et al.*, 1984; Fujii *et al.*, 1985; Hartmans *et al.*, 1985; van Ginkel *et al.*, 1987b), *Nocardia* spp. (Watkinson & Somerville, 1976; Furuhashi *et al.*, 1981; Habets-Crützen *et al.*, 1984; van Ginkel *et al.*, 1987 a & c), *Xanthobacter* spp. (van Ginkel & de Bont, 1986) and a Gram-negative motile rod designated strain PL-1 (Cerniglia *et al.*, 1976).

The first intermediate in the metabolism of gaseous 1-alkenes is the corresponding 1,2-epoxyalkane formed by a NADH-dependent monooxygenase (de Bont *et al.*, 1979 & 1983). The further metabolism of epoxyethane may proceed *via* acetyl-CoA (de Bont & Harder, 1978; Wiegant & de Bont, 1980) and the metabolism of 1,2-epoxypropane *via* a C₂+C₁ cleavage (Cerniglia *et al.*, 1976). A major difference between alkane- and alkene-grown bacteria is the specificity toward gaseous hydrocarbons of the monooxygenases responsible for the initial oxygenation. Alkane monooxygenases from alkane-utilizers can epoxidize olefins as well as hydroxylating 1- and 2-alkenes along with alkanes (May & Abbott, 1973; Hou *et al.*, 1983; van Ginkel *et al.*, 1987c), whereas the alkene monooxygenases

can only epoxidize and do not hydroxylate olefins (Habets-Crützen *et al.*, 1984).

Only a few bacteria that have been isolated on gaseous olefins can also grow on saturated gaseous hydrocarbons. These include *Mycobacterium* sp. E20 (de Bont *et al.*, 1980), *Nocardia corallina* B276 (Furuhashi *et al.*, 1981), *Mycobacterium* spp. H131 & H141 (Fujii *et al.*, 1985), *Nocardia* sp. BT1 (van Ginkel *et al.*, 1987 a) and *Nocardia* sp. TB1 (van Ginkel *et al.*, 1987c).

Mycobacterium E20 contains two monooxygenases (de Bont *et al.*, 1979): an alkene monooxygenase induced by growth of the organism on ethene, produces optically active *R*-1,2-epoxypropane (ee=76%) and *R*-1,2-epoxybutane (ee=62%), while the other enzyme is an alkane monooxygenase induced by growth on ethane, and produces racemic epoxyalkanes (Habets-Crützen *et al.*, 1985). In contrast, *Nocardia corallina* B276 forms chiral *R*-1,2-epoxypropane (ee=74%), *R*-1,2-epoxybutane (ee=68%) and *R*-1,2-epoxytetradecane (ee=86%) when alkane-grown. The enantiomeric composition is not dependent on the substrate used to cultivate the organism (Furuhashi *et al.*, 1981; Weijers *et al.*, 1988 a). It seems therefore, that the same enzyme might be involved in both the hydroxylation and epoxidation of alkanes and 1-alkenes respectively, while the enzyme is also able to epoxidize 2-alkenes (Weijers *et al.*, 1988a). The bacterium grows well on gaseous 1-alkenes as well as long chain olefins (C_{13} - C_{18}), 1,3-butadiene and alkanes (propane, tetradecane). It differs also as it is able to epoxidize 1-alkenes (C_2 - C_{18}) when grown on glucose (Furuhashi *et al.*, 1981 & 1986), whereas the majority of monooxygenases are only induced by alkanes or alkenes. Epoxide production (C_6 - C_{10} 1-alkenes, styrene) is enhanced by the addition of *n*-hexadecane as a solvent, which causes a reduction in substrate/product inhibition in the reaction system (Furuhashi *et al.*, 1986).

Nocardia spp. isolated on 1,3-butadiene (strain BT1) or on isoprene (strain IP1), grow on both of these olefins and butane but not on 1- or 2-alkenes (van Ginkel *et al.*, 1987 a). These organisms form *R*-1,2-epoxypropane (ee=80-86%) and *R*-1,2-epoxybutane (ee=74-98%) stereospecifically also when grown on butane (Weijers *et al.*, 1988 a). A *trans*-2-butene-utilizing *Nocardia* sp. TB1 grows on gaseous alkanes (butane, propane) but

gaseous or volatile 1-alkenes do not support growth. Oxidation of *trans*-2-butene occurs *via* crotonic acid and proceeds *via* β -oxidation (van Ginkel *et al.*, 1987c). The organism is believed to contain an 'alkane-type' monooxygenase that forms *R*-1,2-epoxyalkanes in a very low optical purity (ee=36% for C₃ & 38% for C₄) irrespective of growth substrate (Weijers *et al.*, 1988 a). *Nocardia* spp. B276, BT1 and IP1 are, therefore, clearly distinct from other bacteria because of their ability to form chiral 1,2-epoxyalkanes when grown on alkanes.

Bacteria, able to grow only on olefins (and not on alkanes) form epoxyalkanes stereospecifically, but the enantiomeric composition depends on both the organism used and on the olefin oxidized. Ethene-utilizing *Mycobacteria* form *R*-1,2-epoxypropane (ee=82-86%), *R*-1,2-epoxybutane (ee=66-70%) and *trans*-(2*R*,3*R*)-epoxybutane (ee=56-70%) [Habets-Crützen *et al.*, 1985; Weijers *et al.*, 1988 a]. A recently isolated ethene-utilizing thermophilic organism produces *trans*-2,3-epoxybutane (ee=98%) almost exclusively in the *R*-configuration, whereas the optical purity of 1,2-epoxyalkanes (ee=74% for C₃ & 62% for C₄) is lower than with the mesophilic strains (Weijers *et al.*, 1988 a). Propene- and butene-utilizing *Mycobacterium*, *Nocardia* and *Xanthobacter* spp. also form *trans*-(2*R*,3*R*)-epoxybutane (ee=56-86%) but can only accumulate *R*-1,2-epoxypropane (ee=92-94%) and *R*-1,2-epoxybutane (ee=88-90%) when the further metabolism is inhibited in the presence of excess (10 mM) 1,2-epoxyalkanes as sacrificial substrates (Habets-Crützen *et al.*, 1985; Weijers *et al.*, 1988 a). These bacteria, in general, accumulate epoxyalkanes from non-growth alkenes (van Ginkel *et al.*, 1987b).

Vinyl chloride-utilizing *Mycobacterium* sp. L1 (Hartmans *et al.*, 1985) is distinct from the bacteria isolated on ethene because it forms *R*-1,2-epoxypropane in a very high enantiomeric excess (98%). The enantiomeric composition of the other epoxyalkanes is, however, similar to the mesophilic ethene-utilizing *Mycobacteria* and is only slightly affected by growth substrate (ethene or vinyl chloride). It has been proposed that vinyl chloride, with its bulky chloride group, might impose a constraint on the active site of the enzyme and consequently might have slightly altered its properties in such a way that only

one stereoisomer of 1,2-epoxypropane is formed (Weijers *et al.*, 1988 a).

Pseudomonas sp. (strain H1) isolated on 1-hexene, resembles alkane-utilizing bacteria for i) its ability to grow more abundantly on some alkanes (pentane, hexane) than on 1-hexene and ii) its susceptibility toward epoxides that are formed (1,2-epoxybutane, *cis*-/*trans*-2,3-epoxybutane) [van Ginkel *et al.*, 1987 b]. A 1-hexene-utilizing *Nocardia* sp. (strain H8), however, grows on other 1-alkenes (C_2 - C_3) but is unable to form 1,2-epoxyalkanes (van Ginkel *et al.*, 1987b), whereas *trans*-(2*R*,3*R*)-epoxybutane is formed, although in a very low optical purity (ee=54%) [Weijers *et al.*, 1988 a]. *Corynebacterium equi* IFO 3730 assimilates longer chain terminal olefins (C_{14} - C_{18}) as the sole source of carbon and energy and accumulates the corresponding epoxyalkanes. The optical purity of *R*-1,2-epoxyhexadecane has been determined to be 100% (Ohta & Tetsukawa, 1979).

1.1.4. Nitrifying bacterium

Nitrosomonas europaea contains an ammonia monooxygenase and it has been shown that methane is a substrate for the enzyme (Hyman & Wood, 1983) as is ethene, which is oxidized to epoxyethane (Hyman & Wood, 1984). Epoxyalkanes are formed from 1-alkenes (C_3 - C_5) and *cis*-/*trans*-2-butenes. Alkanes and 1- and 2-alkenes are also hydroxylated (Hyman *et al.*, 1988).

The bacterium catalyzes chiral formation of *S*-1,2-epoxypropane (ee=36%) and *S*-1,2-epoxybutane (ee=42%), whereas all other bacterial epoxide-producing systems form *R*-epoxyalkanes (Weijers *et al.*, 1988 a).

Other interesting epoxidation reactions include the fungal (*Penicillium* spp.) transformation of *cis*-propenylphosphonic acid into (–)-*cis*-1,2-epoxypropylphosphonic acid (fosfomycin, a broad-spectrum antibiotic) [White *et al.*, 1971], bacterial epoxidation of cyclodiene insecticides (e.g. aldrin) and monoterpenes (e.g. α -pinene). Soil bacteria of the genera *Bacillus*, *Mycobacterium*, *Nocardia*, *Streptomyces* and *Pseudomonas* spp. catalyze stereospecific epoxidation of aldrin, and the *exo*-stereoisomer of dieldrin (the

epoxide) is formed exclusively (Ferguson & Korte, 1977). A strain of *Pseudomonas fluorescens* (NCIMB 11671) produces α -pinene oxide in a NADH-linked monooxygenase reaction (Best *et al.*, 1987), while Griffiths *et al.*, (1987) reported that a *Nocardia* sp. (strain P 18.3) when growing on α -pinene oxidizes α -pinene oxide without any lag phase, indicating that the epoxide is an intermediate in the degradation of α -pinene.

1.1.5. Biochemical role of epoxidation reactions

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous environmental pollutants (fig. 2). PAH are present in petroleum, cigarette smoke, automobile exhaust, chimney soot, refuse burning and industrial processes (Blumer, 1976). Considerable attention has been given to the widespread distribution of PAH in the environment and their possible exposure to humans, because many of these compounds are recognized as chemical carcinogens. It has now become evident that PAH undergo metabolic activation that induces toxic, mutagenic or carcinogenic properties (Heidelberger, 1975; Sims & Grover, 1974 & 1981).

The initial stage in the mammalian metabolism of PAH involves the cytochrome P-450 monooxygenase system, which catalyzes the oxidative metabolism of these lipophilic compounds, to form arene oxides (Daly *et al.*, 1972). Arene oxides are relatively unstable and undergo at least five reactions, including: i) non-enzymatic rearrangement to phenols, ii) enzymatic hydration by epoxide hydrolase, to yield *trans*-dihydrodiols, iii) conjugation with glutathione (GSH), either enzymatically by glutathione *S*-epoxide transferase or non-enzymatically, iv) reduction of the arene oxide back to the parent hydrocarbon and v) binding to nucleophilic sites in proteins and nucleic acids (fig. 3). This covalent binding is thought to be a necessary requirement for the initiation of chemical carcinogenesis. The balance between activation and detoxification pathways is important in determining how much of a reactive metabolite will be formed and become available for binding to cellular macromolecules.

Both prokaryotic and eukaryotic microorganisms have the enzymatic capacity to oxidize aromatic substrates. Bacterial oxidations involve incorporation of two atoms of

Figure 2. The structures of selected polycyclic aromatic hydrocarbons .

molecular oxygen into the substrate to form a *cis*-dihydrodiol in a dioxygenase catalyzed reaction (Gibson, 1977). Further oxidation of the *cis*-dihydrodiol leads to the formation of catechols (fig. 4). In contrast to bacteria, fungi oxidize aromatic hydrocarbons *via* cytochrome P-450 monooxygenase and epoxide hydrolase reactions to form *trans*-dihydrodiols (Cerniglia, 1981). These reactions appear to be similar to those reported for mammalian enzyme systems (Gibson & Subramanian, 1984).

Arene oxides are intermediates in the oxidation of naphthalene, anthracene, phenanthrene and derivatives of these compounds by the fungus *Cunninghamella elegans*, which predominately forms *trans*-dihydrodiols. The enzymes involved in these reactions show stereoselective and regioselective properties that are different from the enzymes of mammalian origin (Cerniglia & Yang, 1984; McMillan *et al.*, 1987).

Figure 3. The pathways utilized by mammals for the metabolism of aromatic hydrocarbons. R- aromatic ring (May, 1979).

Figure 4. Pathways of aromatic hydrocarbon oxidation by prokaryotic and eukaryotic microorganisms (May, 1979).

1.1.6. Haloperoxidase & halohydrin epoxidase

Haloperoxidases catalyze the formation of α,β -halohydrins from olefins (C_2 - C_4) in the presence of a halide ion and hydrogen peroxide (Geigert *et al.*, 1983). These enzymes are present in various microorganisms and can be classified as chloroperoxidases (fungi), bromoperoxidases (algae, bacteria) and iodoperoxidases (algae) depending on the smallest halide ion they oxidize (Neidleman & Geigert, 1986). Recently, it has been demonstrated that a chloroperoxidase from *Caldariomyces fumago* is also able to act on olefins in the absence of halide ions (fig. 1) resulting in epoxide formation. The enzyme also catalyzes the epoxidation of propene as well as styrene (Geigert *et al.*, 1986).

Another route for production of epoxides is the enzymatic dehalogenation of α,β -halohydrins (fig. 1). Halohydrin epoxidase isolated from a *Flavobacterium* sp. catalyzes the bioconversion of halohydrins (α,β -chlorohydrins, bromohydrins, iodohydrins) into the corresponding epoxides (Bartnicki & Castro, 1969).

1.2. Microbial metabolism of epoxyalkanes

The different types of epoxide-degrading reactions are summarized in figure 5. Hydrolysis of arene oxides formed during the oxidation of aromatic hydrocarbons in mammalian systems is catalyzed by an epoxide hydrolase, resulting in the formation of *trans*-diols (Gibson & Subramanian, 1984; McMillan *et al.*, 1987). These enzymes are widely distributed in the microsomal fractions of animal tissues, particularly in the liver, and are often highly regio- and enantioselective in their reactions (Seidegard & DePierre, 1983; Berti, 1986; Wistuba & Schurig, 1986). The epoxide hydrolase from *Fusarium solani pisi*, however, catalyzes the *cis*-hydrolysis of epoxyacids (Kolattukudy & Brown, 1975). In mammalian tissue, arene oxides can also undergo conjugation with glutathione (fig. 3) by the action of glutathione *S*-epoxide transferase (Gibson & Subramanian, 1984). Although this enzyme is present in a variety of microorganisms (Lau *et al.*, 1980), no direct proof seems to be available so far for its action in the microbial degradation of arene oxides.

To date, epoxide-degrading ability has only been reported in a few bacteria. This can be partially explained by the inability of methane- and most alkane-utilizing bacteria to degrade epoxides. Degradation of styrene oxide has been investigated in a *Pseudomonas* sp. (strain 305-STR-1-4) that converts styrene oxide into β -phenethyl alcohol (fig. 5) by a styrene oxide reductase (Shirai & Hisatsuka, 1979), while a strain of *Pseudomonas putida* catalyzes the hydrolysis of *trans*-2,3-epoxysuccinate (Allen & Jakoby, 1969). *Nocardia* sp. A60 grows on 1,2-epoxypropane, with 1,2-propanediol as an intermediate, in a reaction catalyzed by an epoxidase without the requirement of a co-factor (de Bont *et al.*, 1982). Alkene-utilizing bacteria also contain an epoxide-degrading enzyme(s). Ethene-grown *Mycobacterium* E20 (de Bont & Harder, 1978) and *Mycobacterium* E44 (Wiegant & de Bont, 1980) catalyze the direct conversion of epoxyethane into acetyl-CoA in the presence of NAD⁺, CoA and an unknown co-factor (fig. 5). α -Pinene-grown cells of *Pseudomonas fluorescens* (Best *et al.*, 1987) and a *Nocardia* sp. strain P18.3 (Griffiths *et al.*, 1987) catalyze the cleavage of both rings of α -pinene oxide (fig. 5), with the formation of a

Figure 5. Reactions involved in microbial epoxide degradation.

branched-chain unsaturated aldehyde (*cis*-2-methyl-5-isopropylhexa-2,5-dienal).

Despite the stereospecificity of microbial epoxidations, production of chiral epoxides on a preparative scale is often hindered due to the fact that the hydrolytic step is usually much faster than the oxidative one (Habets-Crützen *et al.*, 1984; van Ginkel *et al.*, 1986 & 1987b). The enantioselective degradation of racemic epoxyalkanes is a promising alternative procedure for obtaining chiral epoxides. Biological routes to single enantiomer epoxyalkanes involve stereoselective hydrolysis of one stereoisomer while the other isomer would not be affected. Although chiral resolution in this manner involves the destruction of half of the amount of product, it nevertheless may be an economically feasible method, in view of the difference in price between the racemic and optically pure epoxides. Most reports in this field, however, refer to mammalian enzyme systems and only limited attention has been given to microbial enzymes.

Pseudomonas oleovorans degrades racemic 1,2-epoxyoctane stereoselectively, the *R*-enantiomer being hydroxylated and subsequently oxidized faster than the *S*-enantiomer (de Smet *et al.*, 1981a). *Xanthobacter* Py2 can discriminate between enantiomeric forms of 2,3-epoxyalkanes (C₄-C₅), whereas no chiral resolution is observed with 1,2-epoxyalkanes (Weijers *et al.*, 1988 b).

1.3. Applications of epoxidation reactions in biotechnological processes

Monooxygenase-dependent epoxide-producing systems in general, suffer from disadvantages that at present prevent large scale biotechnological production of epoxides. These include i) low specific enzyme activity, ii) toxicity of epoxides toward the organism and iii) the need for co-factor regeneration to replenish reducing equivalents used in the monooxygenase reaction.

Problems associated with *low overall productivity* (Habets-Crützen *et al.*, 1984) of the biocatalyst may be resolved by a) screening for more efficient systems where product degradation is minimal or absent and/or by b) process engineering, e.g., immobilization in multiphase reactors (Brink *et al.*, 1988).

Product inhibition has largely been circumvented by applying water/organic solvent two-phase systems where the solubility of olefins and epoxides in suitable solvents is often much higher than that in water. This makes high concentrations of reactant and product in the bioreactor possible and reduces the effect of any substrate and/or product inhibition in the aqueous biocatalyst phase (Schwartz & McCoy, 1977; de Smet *et al.*, 1983; Harbron *et al.*, 1986; Miyawaki *et al.*, 1986). In the case of volatile or gaseous substrates, a 'gas-solid' bioreactor has been employed consisting of the solid immobilized biocatalyst and a continuous gas phase containing the substrate(s) and the product (de Bont *et al.*, 1983). Sensitivity of the organism to epoxide product and organic solvent is a key parameter in two-liquid phase biocatalysis. Alkene-utilizing bacteria are potentially more suitable for epoxide formation on a large scale than methane- or alkane-utilizers, because the latter are known to be very sensitive to epoxyalkanes (Habets-Crützen & de Bont, 1985).

The type of organic solvent can significantly affect the kinetics and stability of the biocatalyst, the solubility and partitioning of reactants/products, and product recovery and economics of the two-liquid phase biocatalytic process. Introduction of a water-immiscible organic solvent phase causes a rapid inactivation, clotting of biomass and aggregation of

free cell suspensions of ethene-utilizing *Mycobacterium* 2W at the liquid-liquid interface. Immobilization of the cells in hydrophilic gels (calcium alginate, K-carrageenan), however, prevents direct cell-organic solvent contact and provides protection against denaturing solvent effects. High activity retention is favoured by low polar/high molecular weight solvents (Brink & Tramper, 1985). The polarity of the support material can also be decisive for the partitioning of substrates and products among the various phases (Brink *et al.*, 1988). The design of two-liquid phase bioreactors is, therefore, dependent on whether the biocatalyst is free or immobilized, on the choice of organic solvents and on the support material. Mass-transfer resistances, e.g., across the liquid/liquid interface and in the biocatalyst phase can, however, limit the overall reaction rate.

Many investigators have recognized that some type of *co-factor regeneration system* (chemical or enzymatic) will be needed if use of the enzyme is to be economically feasible (fig. 6). This is a crucial factor because the amount of co-factor used is stoichiometric and a very efficient system for regeneration will have to be used unless reductant can be supplied by other means, e.g., *via* an electrochemical system (Kelly & Kirwan, 1977) in which the electrons for the enzymatic reduction are derived from an applied potential.

During the formation of 1,2-epoxyalkanes from olefins by free or immobilized cells, a continuous regeneration of the co-factor NADH is essential. Reduction of NAD⁺ can be achieved through endogenous respiration during a limited period of time, but, for prolonged epoxide formation an external NADH-regenerating co-substrate is required. For instance, epoxyethane formation by immobilized cells of *Mycobacterium* Py1 is promoted by supplying propionaldehyde to the cells (de Bont *et al.*, 1983), while Drozd & Bailey (1984) described *in situ* regeneration of NADH by the co-oxidation of ethene with free cells of *Mycobacterium* NCIB 11626. Hou (1984) reported that 1,2-epoxypropane formation by immobilized cells of methane-utilizing bacteria could be extended after 12 hours of operation through addition of methanol vapour. Ethanol, ethyl acetate and ethene, however, improve prolonged formation of 1,2-epoxypropane by immobilized cells of two (EU1, 2W) strains of ethene-utilizing *Mycobacteria* (Habets-Crützen & de Bont, 1987). The choice of

S : Substrate (e.g. propene)
S-O : Substrate oxidized (e.g. propene oxide)

Figure 6. Co-factor regeneration in a two-enzyme system (Hou, 1984).

a particular electron donor will, therefore, depend on the capacity of the chosen strain to completely oxidize the substrate and also on the bioreactor system employed.

From a microbiological viewpoint, stereospecificity is by far the most important factor in the selection of a suitable biocatalyst for production of optically active epoxides.* The Nippon Mining Company in Japan was the first to announce the production of chiral epoxides from a variety of terminal olefins, through a monooxygenase-type reaction (fig. 7). More recently, a joint venture between Shell & Gist-Brocades (Johnstone *et al.*, 1986) described the stereoselective microbial epoxidation of aryl allyl ethers into (+)-aryl glycidyl ethers, used in the chiral synthesis of *S*-(-) Metoprolol and *S*-(-)-Atenolol (active components of the β -adrenergic receptor blocking drugs).

When considering the production of novel epoxides, it is generally not possible to predict which bacterial strain will produce the product in the highest optical purity. Therefore, a screening programme is necessary to select the best organism for a particular epoxidation reaction. At present, however, commercial production seems possible only when the epoxides produced are optically pure.

The ultimate objective of this project was to obtain a number of biocatalysts, with a broad substrate specificity, as potential epoxide producers in a biotechnological process. Chiral epoxides can be used as synthons in the production of various optically active compounds. Synthesis of non-functionalized chiral epoxides of high enantiomeric excess is difficult to achieve by chemical methods, with the notable exception of asymmetric epoxidation of allylic alcohols (Katsuki & Sharpless, 1980) and the formation of chiral 1,2-epoxypropane (Golding, 1985). Two approaches were undertaken: i) screening for the ability to grow on 'simple' (ethene, propene) olefins on the premise that the enzyme(s) might not be highly substrate specific and ii) screening for organisms that grow on a series of 'blocked' (table 6) olefins. The latter approach was based upon the observation that although primary aliphatic carbons are more readily attacked (in nature) than olefins (Klug & Markovetz, 1971), steric hindrance (e.g. with secondary and tertiary carbons) might reverse this preference, making the attack at the double bond more likely.

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Figure 7. Commercial production of chiral epoxides by Nippon Mining Co. in Japan (advertised in Journal of the American Chemical Society, 1980).

CHAPTER 2

METHODS

2.1. General techniques

2.1.1. Cleaning of glassware

Tubes and glassware to contain media for cultivation and identification of alkene-utilizing bacteria were treated by rinsing with: i) distilled (dist.) water, ii) 20% (V/V) HCl, iii) deionized water (Milli-Q reagent water system, Millipore Ltd.), iv) 10% (W/V) EDTA (pH 8) and v) deionized water and dried in an oven in the inverted position prior to use. This was to ensure removal of any inhibitory substances and free alkali that might be present in new glassware (Cowan & Steel, 1974).

2.1.2. Determination of pH

The pH value of media and buffers was routinely determined by a Data Scientific digital display pH-meter (model PT1-15) equipped with dual automatic and manual temperature compensation.

2.1.3. Sterility

Glassware, media, pipette tips, Eppendorf tubes, tooth picks etc. were sterilized by autoclaving for 15-20 minutes at 121°C (15 psi) in a domestic pressure cooker. Larger volume sterilization was done in an industrial autoclave (Imperial Biotechnology Ltd.). Heat-labile solutions such as amino acids and carbohydrates were filter sterilized (0.22 µm pore size, Millipore).

Liquid olefins were sterilized by passing through chemically resistant filter membranes (AcroLC13, 0.2 µm pore size, Gelman sciences). All sterile transfers were done in a laminar

air flow cabinet (Hepaire Manufacturing Ltd., Essex, England). A unidirectional (laminar) flow of sterile air was made possible by i) use of HEPA (high efficiency particulate air) filters which remove particles down to 0.3 μm and ii) control of the direction of air movement within the chamber by use of perforated baffles.

Sterility of poured agar plates was examined after incubation at 30°C for 24 hours. All plates were pre-dried prior to inoculation. Sterile disposable pipettes (1 & 10 ml), Petri dishes (90 mm diameter) and plastic universal containers (30 ml) were obtained from Sterilin Ltd. Sterile Sabre syringes (1 & 10 ml) were obtained from Gillette Ltd.

2.1.4. Centrifugation

Bacterial suspensions were harvested and washed in a Sorval RC-5 superspeed refrigerated centrifuge. Two rotors were routinely used: GS-3 ($r=5.96$ inch) and SS-34 ($r=4.26$ inch).

Centrifugation of small volumes (e.g. <3 ml) was done on a MSE Micro-Centaur bench centrifuge, the maximum speed achieved being 13,000 g.

2.2. Chemicals

Gaseous alkanes/alkenes (research grade) were obtained from British Oxygen Co., London, UK. Epoxides, liquid alkanes/alkenes and other chemicals (Gold label) were obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorset, UK.

Crystalline bovine serum albumin (RIA grade), Folin reagent and NADH were obtained from Sigma Chemical Co., Surrey, UK. Bacteriological media and reagents for characterization tests and staining materials (Gold label), were obtained from Difco Laboratories, East Molesey, Surrey, UK. Common chemicals (AR grade), dyes and indicators were obtained from BDH Chemicals Ltd., Poole, Dorset, UK.

2.3.

Bacterial strains

Over 100 alkene-utilizing bacteria were isolated in pure culture from a variety of soil, mud and water samples using selective culture techniques. Classification was done according to Bergey's Manual of Systematic Bacteriology (vol. I, 1984; vol. II, 1986). Details of individual strains are given in Chapter 3.

2.4.

Growth conditions

Bacteria were grown at 30°C in a basal mineral-salts (BMS) medium of the following composition (per litre of dist. H₂O):

Fe₂SO₄·7H₂O (1 mg); CuSO₄·5H₂O (5 µg); H₃BO₃ (10 µg); ZnSO₄·7H₂O (70 µg); MnCl₂·4H₂O (13 µg); MgSO₄·7H₂O (0.2 g); CaCl₂·2H₂O (20 mg); NaMoO₄·2H₂O (0.1 ml of 0.1% W/V stock); KNO₃ (2.4 g); Bacto agar (1.6% W/V) for solid medium. Sterile phosphate buffer (pH 7.2) was added after autoclaving to a final concentration of 25 mM [100 ml/l of K₂HPO₄ (44 g); KH₂PO₄ (34 g) stock solution].

Cultivation was done in 2l flasks containing 400 ml BMS medium with 5-10% (V/V) gaseous (ethene, propene) olefins as the sole source of carbon and energy. The inoculum (1-4% V/V) consisted of cells pre-adapted on each substrate.

2.5.

Biomass analyses

2.5.1. Culture turbidity

Optical densities (OD) were routinely measured by a Shimadzu uv-visible recording spectrophotometer. Cell concentrations were determined at a wavelength of 600 nm against appropriate blanks.

2.5.2. Determination of protein

2.5.2.1. *Bio-Rad assay*

(Bradford, 1976)

The concentration of soluble proteins can be determined by the Bio-Rad assay . It is based on the differential colour change of a dye (Coomassie Brilliant Blue G-250) in response to various concentrations of protein. The dye reagent (55% phosphoric acid & methanol) is provided by the manufacturer as a 5 fold concentrate. It is diluted and filtered prior to use in the standard assay procedure. In time, the diluted dye reagent slowly forms a precipitate and is, therefore, prepared in quantities for use only within two weeks.

Soluble subcellular fractions were appropriately diluted with distilled water. Eppendorf tubes containing triplicate samples (5-20 μ l) of soluble fraction, 5-20 μ l of a standard protein solution (BSA, 1 mg/ml) and a blank (20 μ l dist. H₂O) were diluted to 1 ml with the dye reagent. Optical densities (OD_{595 nm}) were measured against the reagent blank after 5 minutes to one hour at room temperature and plotted against the protein concentrations (1-20 μ g) in the standard. Protein content of soluble fractions was calculated from the standard curve.

2.5.2.2. Lowry method

(Lowry *et al.*, 1951)

Reagents:

A.	Sodium bicarbonate in 0.1 N sodium hydroxide	2% (W/V)
B.	Copper sulphate	1% (W/V)
C.	Sodium-potassium tartrate	2% (W/V)
D.	mix (1 ml B + 1 ml C) + 100 ml A discard after one day	
E.	<i>Folin-Ciocalteu</i> reagent	Dilute 1:2 with dist. H ₂ O

Total protein content of whole microbial cells can be determined by extraction and separation of cellular proteins in a soluble form, followed by chemical analysis. Whole cell suspensions were washed (10,800 g at 4°C for 15 min.) twice with deionized water prior to protein estimation, to avoid any interference by culture medium constituents.

Washed-cells (0.5 ml) were diluted with an equal volume of 1N NaOH in Eppendorf tubes and placed in a boiling water bath for 10-15 minutes. The suspension was cooled to room temperature and centrifuged at 13,000 g (MSE bench centrifuge) for 10 minutes to remove insoluble cell-wall components. The resulting supernatant was appropriately diluted with distilled water to a final volume of 1 ml. The diluted protein solution (0.5 ml) was added to 3 ml of reagent D, allowed to stand for 10 minutes at room temperature and *rapidly* mixed with 0.3 ml of reagent E. A blank containing distilled water (0.5 ml), instead of cell suspension, and a set of standard protein solution (BSA, 1 mg/ml) were treated in the same way, including the heating stage. Optical densities (OD_{750nm}) were measured after 30 minutes at room temperature and plotted against the protein concentrations (5-100 µg) in the standard. Total protein was calculated from the standard curve.

2.5.3. Dry weight measurements

(Herbert *et al.*, 1971)

2.5.3.1. Filtration

Washed microbial cells were fixed by adding one part of concentrated formaldehyde solution (40% V/V) directly to 9 parts of culture. This provided an effective way to stop cellular growth and allowed a reliable and precise determination of the dry weight. Filtration was the chosen method with dilute samples because filtering speed became slow when too many cells were present. Dried pre-weighed Millipore filters (0.45 μm pore size) were used to filter 5-10 ml of bacterial suspensions. The collected cells were washed twice with deionized water. Filters were dried for 3 days at 70°C and weighed within 15 minutes of removal from the oven.

2.5.3.2. Centrifugation

Bacterial suspensions were centrifuged, the supernatant removed by decantation and cells resuspended in an equal volume of deionized water. The washing procedure was repeated twice. Washed-cell suspensions (10-40 ml) were fixed with concentrated formaldehyde solution (40% V/V), poured into dried pre-weighed pans and placed in an oven at 70°C for 3 days. The pans were then allowed to cool to room temperature in a desiccator, and were weighed within a few minutes of removal from it. This method was mostly used with relatively dense cell suspensions.

2.6. Bioconversions

Cells were harvested during exponential growth by centrifugation (10,800 g at 4°C for 30 min.), washed twice with 25 mM phosphate buffer (pH 7) and resuspended in fresh buffer ('resting-cells'). Washed-cell suspension of each bacterium (1-4 g/l DW) was pre-incubated for 5 minutes in either 7 ml stoppered flasks (for gaseous hydrocarbons) or in 100 ml screw-capped conical flasks (for liquid hydrocarbons). Each substrate was tested at

various concentrations to determine the optimum conditions for its oxidation.

The bioconversion was started by injecting gaseous olefins (10-40% V/V) displacing an equal volume of air, whereas liquid olefins were added to a final concentration of 1-5 mM. All incubations were done in triplicate at 30°C (except otherwise stated) in a water bath rotary shaker at 300 rpm (New Brunswick Scientific Co., Edison, N.J.). Control experiments were done with heat-killed cells (15 min. at 80°C) and also with reaction mixtures lacking oxygen or substrate.

2.7. Preparation of cell-free extracts

Ethene-grown 'resting-cells' of *Micrococcus* sp. M90C were divided into two aliquots, with or without the addition of β -mercaptoethanol (5 mM). These were disrupted by two passages through a French pressure cell (American Instruments Co., Silver Spring, Md.) at 20,000 psi and centrifuged at 7700 g for 20 minutes (4°C) to remove unbroken bacteria and cell debris. The supernatant solution (crude extract) was then centrifuged at 48,000 g for 90 minutes (4°C), yielding particulate (P48) and soluble (S48) fractions. Particulate fractions were washed and resuspended in a minimal volume of phosphate buffer (25 mM, pH 7). Fresh subcellular fractions were assayed for epoxidation activity from a range of gaseous (C_2 - C_4) olefins (fig. 8).

Assays were done in triplicate at 30°C in 7ml stoppered flasks containing 1ml of reaction mixtures: 0.95ml of subcellular fractions and 5 μ mole of NADH (in those reactions without NADH, 1ml of subcellular fractions were used). The bioconversion was started by injecting gaseous olefins (10-40% V/V).

2.8.

Product analyses

2.8.1. Gas liquid chromatography

C₂-C₄ Epoxides: Aqueous samples (1 µl) were directly analyzed (without extraction) by flame ionization (FID) gas chromatography (Philips, Pu 4500) on a glass column packed with Porapak Q (0.9 m x 4 mm id.) at 160°C (isothermal) with N₂ carrier gas at 40 ml/min. (Appendix). Epoxide concentration was calculated from peak areas using standard curves (external standardization method).

C₅-C₁₆ Epoxides & phenyl methyl sulphoxide: Aqueous samples (50 µl) were periodically removed from reaction flasks and extracted into an equal volume of ice-cold ether (complete extraction of substrates and products was achieved) containing 1-alkenes as internal standards (Appendix). The 'cocktail' was shaken vigorously for 1 minute, centrifuged at 13,000 g for 10 minutes, cooled and samples (1 µl) taken from the top ether layer for analysis. Quantitation was done by peak area integration (internal standardization method) using a Shimadzu Chromatopac CR-6A integrator.

Structure identification and purity of the product were done by retention time comparisons, co-chromatography with authentic standards, electron impact mass spectrometry, chiral complexation capillary glc, ¹H NMR using the europium shift reagent tris-[heptafluoropropyl hydroxymethylene-d-camphorato]-europium (III), Eu(hfc)₃, and HPLC. Identification of microbial epoxides was confirmed by treatment of reaction mixtures with 10-20 µl of concentrated H₂SO₄, followed by gas chromatography, to establish presence or absence of epoxide peak after acid hydrolysis (May & Abbott, 1973). Complete hydrolysis of epoxide occurred at room temperature within 5-10 minutes after addition of the acid.

2.8.2. Mass spectrometry

Structures of phenyl methyl sulphoxide and phenyl glycidyl ether were confirmed by electron impact mass spectrometry (Perkin-Elmer sigma-3 GC-MS 25 Kratos). *Gas chromatograph*: SGE-fused vitreous silica capillary column (25 m x 0.25 mm id.) with splitless injection; injection temperature, 225°C; temperature programme, 100°C (2 min.) at 15°C/min. to 250°C. *Mass spectrometer*: source temperature, 200°C; accelerating voltage, 2Kv; ionization energy; 70eV; trap current, 100 µA.

Data was acquired on a Kratos DS-55 computer data system.

2.9. Chiral analyses

2.9.1. Chiral complexation capillary glc

The enantiomeric composition of microbially produced 1,2-epoxypropane, 1,2-epoxybutane and *trans*-2,3-epoxybutane was determined by chiral complexation glc (Schurig & Bürkle, 1982).

Gaseous samples (100-200 µl) were removed using a gas-tight syringe (Precision Sampling Corp., USA). Analyses were done on a Packard model 436 gas chromatograph equipped with a variable split. A glass capillary column (50 m x 0.25 mm id.) coated with 0.12 M - Ni(II)-bis-(3-heptafluorobutyryl-1*R*-camphorate) in OV101 was used. The oven temperature was 70°C with N₂ carrier gas at 0.4 ml/min. Split ratio was 1:50.

2.9.2. Chiral-shift ¹H NMR

To produce sufficient material for chiral analyses the bioconversion was scaled-up, yielding up to 20 mg of phenyl methyl sulphoxide and 54 mg of phenyl glycidyl ether (1,2-epoxy-3-phenoxy propane). After removal of the cells by centrifugation, the broth was extracted twice with an equal volume of ether. The combined ether layers were washed once with distilled water (pH 7-8), dried over Na₂SO₄ and the ether removed by rotary

evaporation. The enantiomeric purity of products was determined by ^1H NMR spectrometry (Bruker 200 or 270 MHz) by adding the chiral-shift reagent (CSR), $\text{Eu}(\text{hfc})_3$, to the sulphoxide dissolved in benzene- d_6 (0.2 molar equivalent of CSR) or to the epoxide dissolved in acetone- d_6 (0.125 molar equivalent of CSR).

2.9.3. HPLC

Further confirmation of the optical purity of microbial phenyl glycidyl ether was established by HPLC analysis (Waters Associates equipped with a Kontron autosampler). Analytical conditions were: CHIRALCEL-OD column (25 cm x 0.46 cm id.); mobile phase, 50% isopropyl alcohol/50% hexane (V/V); flow rate, 0.5 ml/min.; injection volume, 10 μl ; detection wavelength, 280 nm; pressure, 500 psi.

Data was acquired on a Nelson chromatography software (version 3.6) on an IBM PC-XT.

2.10. Optical rotation

Optical rotation measurements were done on a Perkin-Elmer 141 polarimeter.

2.11.

Staining

All media and reagents for staining and characterization tests were freshly prepared according to Manual for Identification of Medical Bacteria (1974) and Difco Manual (1984) and kept in well-sealed glass-stoppered bottles protected, from direct sunlight.

2.11.1. Differential stains

2.11.1.1. *Gram method*

Crystal violet stain:	Bacto crystal violet	1% (W/V)
Decolourizer:	Acetone	250 ml
	Isopropyl alcohol	750 ml
Gram iodine:	Iodine crystals	1g
	Potassium iodide	2g
	Dist. H ₂ O	300 ml
Gram safranin:	Safranin powder	4g
	Alcohol, anhydrous	200 ml
	Dist. H ₂ O	800 ml

Preparation of smears from cultures

For microorganisms in liquid suspension:

- 1) Spread a loopful on a clean glass slide.
- 2) Let smear air-dry.
- 3) Heat-fix by passing through a low gas flame 3 times.
- 4) Allow fixed slide to cool to room temperature before staining.

For staining microorganisms from colonies on solid media:

- 1) Place a small loopful of distilled water on a clean microscope slide.
- 2) Touch a sterile inoculating loop to a colony, rub it into the droplet to obtain an even suspension of microorganisms and spread it over the surface of the slide.
- 3) Let the smear dry at room temperature.
- 4) Heat-fix by passing the slide 3 times through a low gas flame.

5) Cool to room temperature before staining.

Method of staining:

- 1) Flood the fixed smears with Gram crystal violet stain and allow to stain for 1 minute.
- 2) Wash off excess crystal violet lightly with cold tap water.
- 3) Flood slide with Gram iodine and allow the iodine to remain on slide for 1 minute.
- 4) Remove iodine by gentle washing with tap water.
- 5) Flood off excess water with Gram decolourizer until solvent runs colourlessly from the slide (10-15 seconds).
- 6) Wash slide gently and thoroughly in cold tap water.
- 7) Counterstain with safranin for 45 seconds.
- 8) Wash off excess stain with cold tap water and stand on end to drain.
- 9) Examine under an oil immersion lens.

Interpretation:

Gram-positive organisms are blue or purple. Cells which decolourize and stain pink to red with counterstain are Gram-negative. For best result 18-24 hour cultures were used, since fresh cells have a greater affinity than old cells for most dyes.

2.11.1.2. Ziehl-Neelsen method (Acid-fast stain)

Carbol fuchsin, strong: *Solution A*

For use, pour 10 ml	Basic fuchsin	10 g
of solution A into	Ethanol (95%)	100 ml
100 ml of solution B.		

Solution B

Phenol	5 g
Dist. H ₂ O	100 ml

Acid-alcohol:	conc. HCl	3 ml
	Ethanol (95%)	97 ml

Counterstain:	Malachite green	0.5% (W/V)

Method of staining:

- 1) Flood the slide with strong carbol fuchsin and heat until steam rises (but do not boil).
- 2) After 3-4 minutes apply more heat until steam rises again, but do not let the stain dry on the slide.
- 3) About 5-7 minutes after the first application of heat, wash the slides thoroughly under running water.

- 4) Decolourize in acid-alcohol until all traces of red have disappeared from the film.
Decolourization should be done by intermittent washings in water and re-application of acid-alcohol (10-20 seconds).
- 5) Wash well in water when decolourization is complete.
- 6) Counterstain with malachite green for 1 minute.
- 7) Wash and stand on end to drain (do not blot).

Interpretation:

Acid-fast organisms are red, others green.

2.11.2. Special stains

2.11.2.1. Spore stain

Loeffler methylene blue: *Saturated solution*

Mix in order; bottles should be shaken frequently before use. The stain improves with keeping.

Methylene blue ..... 1 g
Ethanol (95%) ..... 100 ml

Staining solution

KOH 1% aq. solution ..... 1 ml
Dist. H₂O ..... 99 ml
Ethanol methylene blue ..... 30 ml

Method of staining:

This is similar to Ziehl-Neelsen method but ethanol is used for decolourization.

- 1) Flood the slide with strong carbol fuchsin and steam.
- 2) After 5 minutes wash well in water.
- 3) Decolourize with ethanol until all traces of red have been removed.
- 4) Wash thoroughly in water.
- 5) Counterstain with Loeffler methylene blue for 1-2 minutes.
- 6) Wash and drain to dry.

Interpretation:

Bacterial bodies stain blue, spores red.

2.11.2.2. Capsule stain

Leifson stain:	KAl (SO ₄) ₂ . 12H ₂ O (sat. aq. soln.)	20 ml
	Tannic acid (20% aq. soln.)	10 ml
Mix ingredients in named order.	Dist. H ₂ O	10 ml
	Ethyl alcohol (95%)	15 ml
	Basic fuchsin (sat. soln. in 95% ethyl alcohol)	3 ml
Counterstain:	Methylene blue	0.1g
	Borax (Na ₂ B ₄ O ₇ .10H ₂ O)	1g
	Dist. H ₂ O	100 ml

Method of staining:

- 1) Flood the slide with Leifson stain and allow to stand for 10 minutes at room temperature.
- 2) Wash in tap water.
- 3) Counterstain with borax methylene blue for 5-10 minutes without heating.
- 4) Wash and drain to dry.

Interpretation:

Capsules stain red, cells blue.

2.12.

Characterization tests

Unless otherwise indicated, cultures were incubated at 30°C or at their optimum temperature as required. All media used for identification were incubated 24 hours before use to ensure sterility. Inoculum consisted of 18-24 hour grown pure cultures of the test organisms on nutrient agar/broth, or other suitably enriched media, e.g., peptone water or glucose broth.

Nutrient broth

Beef extract ..... 3 g
Peptone ..... 5 g
Dist. H₂O ..... 1000 ml
Final pH 6.8 at 25°C

Nutrient agar

This is essentially nutrient broth gelled by the addition of 1.5% (W/V) Bacto agar.

Peptone water

Peptone ..... 10 g
NaCl ..... 5 g
H₂O ..... 1000 ml

Glucose broth

Glucose (20% aq. soln.) ..... 50 ml
Nutrient broth ..... 950 ml

Sterilize the glucose by filtration and add aseptically to nutrient broth.

2.12.1. Arginine hydrolysis

To measure the enzymatic ability of an organism to decarboxylate arginine to form an amine, with resulting alkalinity.

Arginine broth:

Peptone ..... 5 g
Yeast extract ..... 5 g
Dipotassium phosphate ..... 2 g
Glucose ..... 0.5 g
Arginine monohydrochloride .... 3 g
Dist. H₂O 1000ml

Nessler reagent:

- 1) Dissolve 5 g of potassium iodide in 5 ml of distilled water.
- 2) Add cold saturated mercuric chloride solution until a slight precipitate remains after shaking.
- 3) Add 40 ml of 9N NaOH.
- 4) Dilute with distilled water to 100 ml.
- 5) Allow to stand for 24 hours before use.

Procedure:

- 1) Dissolve each component of arginine broth by gentle heating (adjust to pH 7).
- 2) Dispense approximately 5 ml per screw-cap tube (16 x 125 mm).
- 3) Sterilize in the autoclave for 15 minutes at 121°C and cool.
- 4) Use a light inoculum; run a control tube, containing no arginine, with each organism.
- 5) After 24 hour incubation add 0.25 ml Nessler reagent; run the same check on the control.

Interpretation:

Arginine hydrolysis is indicated by the development of a brown colour.

2.12.2. Bile esculin (aesculin) hydrolysis

To determine the ability of an organism to hydrolyze the glycoside esculin to esculetin and glucose in the presence of bile.

Beef extract	3 g
Peptone	5 g
Oxgall (Bile)	40 g
Esculin	1 g
Ferric citrate ($\text{FeC}_6\text{H}_5\text{O}_7$)	0.5 g
Bacto agar	15 g
Dist. H_2O	1000 ml

Final pH 6.6 at 25°C

Procedure:

- 1) Suspend the appropriate amount in 1 litre of distilled water and heat *gently* to boiling to dissolve completely.
- 2) Dispense approximately 5 ml per screw-cap tube.
- 3) Sterilize in the autoclave for 15 minutes at 121°C. Overheating may cause darkening of the media.
- 4) Cool in a slanted position (long slants).
- 5) Inoculate by making a single streak and incubate.

Interpretation:

- A. Positive; presence of a dark brown to black colour on the slant.
Half or more of the medium is blackened at any time interval.
- B. Negative;
- 1) No blackening of the medium.
  - 2) Blackening of less than half of the tube after 72 hours ($\pm$ reaction).
  - 3) Growth may occur but this does not indicate esculin hydrolysis;
only indicates that the bile concentration does not inhibit usual growth.

2.12.3. Carbohydrate fermentation

To determine the ability of an organism to ferment (degrade) a specific carbohydrate, incorporated in a basal medium, producing acid or acid with visible gas.

Phenol red broth base (pH 7.4)

Peptone	10 g
Beef extract	1 g
NaCl.	5 g
Phenol red	0.02 g
Dist. H ₂ O	1000 ml

pH Indicator (Phenol red):

Acid; yellow (pH 6.8)
Alkaline; pinkish-red (pH 8.4)
Uninoculated medium; reddish-orange (pH 7.4)

Procedure:

- 1) Weigh out amounts accurately, rehydrate with distilled water and heat gently into solution.
- 2) Sterilize in the autoclave for 15 minutes at 121°C, cool to 45-50°C in a water bath.
- 3) Filter sterilize individual carbohydrates and aseptically transfer to phenol red broth base to a final concentration of 0.5-1% (W/V).
- 4) Aseptically dispense approximately 5 ml in sterile tubes.
- 5) Place sterile Durham insert in the carbohydrate tubes in an *inverted* position.
- 6) Inoculate by aseptically dipping a loopful of the tested organism into each tube and incubate.

A variety of carbohydrates may be employed depending on the difficulties encountered in identifying a particular organism. The carbohydrates used were glucose, lactose, mannitol, sucrose or xylose.

Interpretation:

A. Positive; yellow (acidic).

- 1) Positive for gas production; gas bubble(s) present in Durham insert or medium is completely displaced by gas, leaving a colourless portion in insert.
- 2) Negative for gas production; no gas bubble(s) present and the medium in Durham insert remains yellow.

B. Negative; reddish-pink (alkaline).

2.12.4. Catalase activity

To test for the presence of the enzyme catalase.

Hydrogen peroxide (H_2O_2) . . . . . 3% aq. soln.
(refrigerate)

Procedure:

- 1) With an inoculating needle, pick the centre of a 18-24 hour pure colony and place on a clean glass slide.
- 2) Add a drop of 3% (V/V) H_2O_2 over the organism on slide. Mixing of culture and H_2O_2 is not necessary.

Interpretation:

- A. Positive; immediate bubbling (O_2 formed).
B. Negative; no bubbling.

2.12.5. Citrate utilization

To determine if an organism is capable of utilizing citrate as the sole source of carbon for metabolism, with resulting alkalinity.

Magnesium sulphate	0.2 g
Ammonium dihydrogen phosphate	1 g
Dipotassium phosphate	1 g
Sodium citrate	2 g
Sodium chloride	5 g
Bacto agar	15 g
Bromothymol blue	0.08 g
Dist. H ₂ O	1000 ml

Final pH 6.8 at 25°C

pH Indicator (Bromothymol blue):

Acid; yellow (pH 6)

Alkaline; deep Prussian blue (pH 7.6)

Uninoculated medium; green (pH 6.9)

Procedure:

- 1) Rehydrate with distilled water and heat to boiling to dissolve completely.
- 2) Place approximately 5 ml in each tube.
- 3) Sterilize in the autoclave for 15 minutes at 121°C.
- 4) Allow medium to solidify in a slanted position (long slant, short butt).
- 5) Use a light inoculum, streak and incubate.

Interpretation:

- A. Positive; growth with an intense blue colour on the slant.
- B. Negative; no growth with no colour change (green).

2.12.6. Fluorescein production

To determine production of Fluorescein pigment by members of *Pseudomonas* spp.

King, Ward & Raney medium B:

Peptone	20 g
Glycerol	10 g
Dipotassium phosphate	1.5 g
Magnesium sulphate	1.5 g
Dist. H ₂ O	1000 ml

Procedure:

- 1) Dissolve each component, by gentle heating into solution (pH 7.2).
- 2) Dissolve 15 g of Bacto agar by boiling and sterilize in the autoclave for 15 minutes at 121°C.
- 3) Pour into individual Petri dishes, cool and inoculate (24 hr. incubation).
- 4) Examine for the presence of Fluorescein.

Interpretation:

- A. Positive; fluorescence under an infra-red or natural light.
B. Negative; no fluorescence.

2.12.7. Gelatin liquefaction

To determine the ability of an organism to produce proteolytic-like enzymes (gelatinases) which liquefy gelatin.

Nutrient gelatin:	Beef extract	3 g
	Peptone	5 g
	Gelatin	120 g
	Dist. H ₂ O	1000 ml

Procedure:

- 1) Add gelatin to distilled water and let stand for 15-30 minutes.
- 2) Heat to dissolve the gelatin, add and dissolve the other components (do not overheat).
- 3) Adjust pH to 7.0 and dispense approximately 5 ml per screw-cap tube.
- 4) Sterilize by autoclaving for 15 minutes at 121°C.
- 5) Cool in an upright position, use a heavy inoculum and stab medium to a depth of 0.5-1 inch.
- 6) Set up a control tube (uninoculated) to run alongside the tested organism.
- 7) Incubate for up to 14 days; every 2-3 days cool in a refrigerator for 2 hours and examine for liquefaction.

Interpretation:

- A. Positive; medium liquefied (test organism).
- B. Negative; medium remains solid, re-incubate for an additional period.
- C. Control tube; medium remains solid.

2.12.8. Indole production

To determine the ability of an organism to split indole from the tryptophan molecule.

Kováč reagent:	<i>p</i> -Dimethylaminobenzaldehyde	5 g
	Amyl alcohol	75 ml
	conc. HCl	25 ml

Dissolve the aldehyde in the alcohol by gently warming in a water bath (about 50-55°C). Cool and slowly add the acid (refrigerate). The reagent should be light yellow to light brown in colour.

Procedure:

- 1) Prepare peptone water and dispense approximately 5 ml per tube.
- 2) Sterilize by autoclaving for 15 minutes at 121°C and cool.
- 3) Use a light inoculum and incubate for 24-48 hours.
- 4) Add 5 drops of Kovác reagent directly to each tube, shake gently and examine after 1 minute.

Interpretation:

- A. Positive; a red ring at the surface of the medium, in the alcoholic layer indicates indole.
- B. Negative; no colour development at the alcohol layer, takes the colour of the Kovác reagent (yellow).
- C. Variable ($\pm$); an orange colour at the surface of the medium, due to the development of a methylated compound (skatole). This is believed to be a precursor for indole formation.

2.12.9. MacConkey agar

A medium used in the differentiation of lactose fermenting organisms from non-lactose fermenting Gram-negative bacteria. Gram-positive bacteria are inhibited, due to the presence of crystal violet and bile salts.

Bacto MacConkey agar

Peptone	20 g
Lactose	10 g
Bile salts	1.5 g
NaCl	5 g
Neutral red	0.03 g
Crystal violet	0.001 g
Bacto agar	13.5 g

Final pH 7.1 at 25 °C

Procedure:

- 1) Rehydrate in 1 litre of distilled water. Heat to boiling with gentle swirling to dissolve completely.
- 2) Sterilize in the autoclave for 15 minutes at 121°C. Avoid overheating of the medium.
- 3) Cool to 45-50°C, dispense into sterile Petri dishes and dry.
- 4) Streak a light inoculum and incubate; examine for growth after 18-24 hours incubation.

Interpretation:

A. Positive; growth.

- 1) Lactose fermentors (LF), colonies of organisms capable of fermenting lactose produce a localized pH drop which (followed by absorption of the neutral red) imparts a red colour to the colony. A zone of precipitated bile may also be present due to this localized drop in pH.
- 2) Non-lactose fermentors (NLF), colonies of organisms which do not ferment lactose remain colourless and translucent.

B. Negative; no growth.

2.12.10. Methyl red (MR)

To test the ability of an organism to produce and maintain stable acid end-products from glucose fermentation and to overcome the buffering capacity of the system.

Glucose-phosphate medium:	Peptone	5 g
	Dipotassium phosphate	5 g
	Glucose	5 g
	Dist. H ₂ O	1000 ml
Methyl red solution: Dissolve methyl red in ethanol and dilute to volume with water.	Methyl red	0.04 g
	Ethanol (95%)	40 ml
	Dist. H ₂ O	60 ml

Procedure:

- 1) Dissolve each component of glucose-phosphate medium by gentle heating.
- 2) Dispense approximately 5 ml per tube.
- 3) Sterilize by autoclaving for 15 minutes at 121°C and cool.
- 4) Use a light inoculum and incubate for 3-5 days.
- 5) Add methyl red pH indicator (2 drops) directly to an incubated aliquot, shake and examine.

Interpretation:

- A. Positive; culture is sufficiently acid to allow the methyl red reagent to remain a distinct red colour (pH 4.4) at the surface of the medium.
- B. Negative; yellow colour (pH 6) at the surface of the medium.
- C. Delayed reaction; orange colour, continue incubation to 4 days and repeat the test.

2.12.11. Motility

To determine if an organism is motile or non-motile.

Tryptose	10 g
NaCl	5 g
Bacto agar	5 g
Dist. H ₂ O	1000 ml

Final pH 7.2 at 25 °C

Procedure:

- 1) Weigh out amounts accurately; rehydrate with distilled water and heat gently into solution.
- 2) Dispense approximately 5 ml into tubes and sterilize in the autoclave for 15 minutes at 121°C.
- 3) Allow medium to cool in an upright position.
- 4) Inoculate by stabbing through the centre of the medium to a depth of 0.5 inch.
- 5) Incubate and examine at 18 and 40 hours; if negative, incubate (21-25°C) at a lower temperature for 5 days. The incubation temperature is crucial, as many motile organisms are motile at 21-25°C but not at their optimum growth temperature.

Interpretation:

- A. Positive (motile); motile organisms migrate from the stab line and diffuse into the medium causing a turbidity. They may also exhibit fuzzy streaks of growth.
- B. Negative (non-motile); growth accentuated along the stab line, the surrounding medium remains clear.
- C. Uninoculated medium; no growth, medium remains colourless and clear.

Note: As the motility test is an important criterion for identification of any organism, all the cultures were, therefore, re-examined in 'hanging-drop' preparations.

'Hanging-drop' preparations:

Young broth cultures of each organism, incubated at or below the optimum temperature, were examined for motility in 'hanging-drop' preparations by phase-contrast microscopy (Zeiss microscope).

A drop of culture was put on a coverslip and covered by a slide bearing a concavity. The slide was pressed gently against the coverslip and turned over quickly, to ensure that the drop remained 'hanging' from the coverslip in the centre of the concavity. The glass was made water-repellent by rubbing it with a lens tissue. Motile species travelled at various rates across the field, allowing the flagellar movement to be seen.

2.12.12. Nitrate reduction

To determine the ability of an organism to reduce nitrate to nitrites or free nitrogen gas.

Nitrate broth

Beef extract	3 g
Peptone	5 g
Potassium nitrate	1 g
Bacto agar	4 g
Dist. H ₂ O	1000 ml

Final pH 7.0 at 25 °C

Reagent A:	α-Naphthylamine	5 g
	Acetic acid (5N)	1000 ml
Reagent B:	Sulphanilic acid	8 g
	(<i>p</i> -aminobenzene sulphonic acid) Acetic acid (5N)	1000 ml

Procedure:

- 1) Dissolve each component of nitrate broth by gentle heating.
- 2) Dispense approximately 5 ml per tube. Place Durham insert in the nitrate tubes in an *inverted* position. Sterilize in the autoclave for 15 minutes at 121°C.
- 3) Inoculate and incubate for 12-24 hours; prolonged incubation may be required.
- 4) Two controls: Tube 1, inoculate with a known nitrate-positive organism. Tube 2, uninoculated but run under the same conditions as if inoculated.

Interpretation:

A. Check for gas production.

- 1) If gas is present (gas bubbles in Durham insert) and the organism is a non-fermentor, test is positive for denitrification (production of N_2 or N_2O or non-gaseous breakdown products, other than NO_2^-).
- 2) If gas is present and the organism is a fermentor, proceed to Phase 1 (gas is hydrogen).
- 3) If there is no gas, proceed to Phase 1.

B. Phase 1.

- 1) To an incubated nitrate culture directly add reagents. Immediately before testing, mix equal parts of reagent A and B. Add approximately 10 drops of the mixture to the culture and shake gently to mix reagents.
- 2) A positive result (red colour) within 1-2 minutes denotes a completed test, i.e., nitrate (NO_3^-) is reduced to nitrite (NO_2^-) by the organism.
- 3) If negative (no colour development) then proceed to Phase 2.

C. Phase 2. Zinc reduction method.

- 1) To the tube containing reagents A and B, directly add a pinch of zinc dust (must be nitrate-nitrite free).
- 2) Positive test (no colour development) denotes absence of nitrite in the medium, i.e., organism reduced $NO_3^- \rightarrow NO_2^- \rightarrow$ non-gaseous products (denitrification). Test for ammonia by adding a few drops of Nessler reagent (positive reaction, a deep orange colour).
- 3) Negative test (pink to a deep red colour within 5-10 minutes) denotes presence of nitrate and is not reduced by the organism (zinc reduced nitrate to nitrite).

2.12.13. Oxidase activity

To determine the presence of the oxidase enzymes.

Tetramethyl-*p*-phenylenediamine dihydrogen chloride . . . . . 1% aq. soln.

Colourless; autooxidation may be retarded
by the addition of 1% ascorbic acid.
The solution should not be used if it becomes
deep blue.

Procedure:

- 1) Place a piece of filter paper in a Petri dish.
- 2) Add 2-3 drops of reagent to the centre of the paper (do not allow drops to dry).
- 3) With a *platinum* inoculating needle (not nichrome), smear a loopful of freshly grown colony onto the reagent-impregnated paper and examine within 5-10 seconds.

Interpretation:

Positive; dark purple colour development within 10 seconds.

2.12.14. Oxidation fermentation (OF)

To determine the oxidative or fermentative metabolism of a carbohydrate or its non-utilization.

**Hugh & Leifson OF basal medium
for Gram-negative organisms**

Tryptone	2 g
NaCl	5 g
Dipotassium phosphate	0.3 g
Bromothymol blue	0.08 g
Bacto agar	2 g
Dist. H ₂ O	1000 ml

pH Indicator (Bromothymol blue):

Acid; yellow (pH 6)

Alkaline; deep Prussian blue (pH 7.6)

Uninoculated medium; green (pH 7.1)

**Fermentation medium for Gram-
positive organisms**

Tryptone	10 g
Yeast extract	1 g
Bromocresol purple	0.01 g
Bacto agar	2 g
Dist. H ₂ O	1000 ml

pH Indicator (Bromocresol purple):

Acid; yellow (pH 5.2)

Alkaline; purple (pH 6.8)

Uninoculated medium; purple (pH 7)

Procedure:

- 1) Weigh out amounts accurately, rehydrate with distilled water and gently heat into solution.
- 2) Sterilize in the autoclave for 15 minutes at 121°C and cool to 40-45°C in a water bath.
- 3) Filter sterilize carbohydrates (glucose, lactose) and aseptically add to the basal medium to a final concentration of 0.5-1% (V/V).
- 4) Aseptically dispense approximately 5 ml per sterile screw-cap tube.
- 5) For each carbohydrate used, inoculate a pair of OF tubes for each organism being tested (label one open & the other sealed). Also set up control sets; one inoculated set with no carbohydrate added, and one uninoculated set with carbohydrate.
- 6) Inoculate by stabbing both tubes to approximately 0.25 inch from the bottom.
- 7) To all tubes labelled as sealed, overlay with approximately 1-2 ml of sterile melted petrolatum (mineral oil) to exclude all oxygen.
- 8) Incubate for 48 hours or longer.

Interpretation:

Table 4. Carbohydrate utilization pattern for Gram-negative organisms.

Reaction	Tube with reaction	Open tube	Sealed tube
Oxidation (O)	Open	Yellow (A)	Green (–)
Fermentation (F)	Sealed	Yellow (A or AG)	Yellow (A or AG)
Neither (Inert)	Uninoculated, carbohydrate control (no colour change)	Blue or green (–)	Green (–)
Both (O) & (F)	Both	Yellow (A or AG)	Yellow (A or AG)

A : Acid production

AG : Acid and gas production

(–) : No change (alkaline reaction)

Table 5. Carbohydrate utilization pattern for Gram-positive organisms.

Reaction	Tube with reaction	Open tube	Sealed tube
Oxidation	Open	Yellow (A)	Purple (–)
Fermentation	Sealed	Yellow (A)	Yellow (A)
Inert	Uninoculated, carbohydrate control (no colour change)	Purple (–)	Purple (–)

A : Acid production

(–) : No change (alkaline reaction)

2.12.15. Slide Culture

To aid differentiation between *Nocardia* and *Streptomyces* spp.

Glucose	40 g
Peptone	10 g
Pancreatic digest of casein	5 g
Bacto agar	16 g
Dist. H ₂ O	1000 ml

Procedure:

- 1) Aseptically cut small blocks of medium (1 × 1 cm) from a poured plate. Place a block of agar onto the centre of a sterile slide.
- 2) Inoculate the edges of each block and apply a coverslip.
- 3) Make a moist chamber by cutting two lengths from a plastic drinking straw and glueing them (5 cm apart) to the bottom of a plastic Petri dish with sterile water (approx. 8 ml).
- 4) Incubate and watch the progress of growth using the low power of the microscope when visible growth has developed.
- 5) When typical structures are seen, prepare a slide and a coverslip, each with a drop of mountant (10% W/V glycerol). Lift the coverslip off the slide culture and mount on the prepared slide, then lift off and discard the agar block; the slide is now mounted on the prepared coverslip.
- 6) Examine both preparations after 30 minutes.

Interpretation:

Nocardia spp. show mycelial forms and primary branching in young cultures which easily fragment in older cultures to coccoid and bacillary forms, whereas *Streptomyces* spp. do not fragment.

2.12.16. Starch hydrolysis

To determine the ability of an organism to hydrolyze starch by enzymatic action, and also to test for the disappearance of starch by use of Gram iodine reagent.

Bacto starch agar

Beef extract	3 g
Soluble starch (Difco)	10 g
Bacto agar	12 g
Dist. H ₂ O	1000 ml

Final pH 7.5 at 25°C

Procedure:

- 1) Rehydrate with distilled water and gently heat to dissolve completely.
- 2) Sterilize in the autoclave for 15 minutes at 121°C.
- 3) Pour into individual Petri dishes, cool and inoculate.
- 4) Flood the surface of a 48 hour culture with Gram iodine reagent and interpret immediately, as blue colour formed with starch may fade.

Interpretation:

- A. Positive; starch hydrolysis, medium purple-blue with a *colourless* area (hydrolysis zone) around growth.
- B. Negative; starch not hydrolyzed, medium purple-blue colour right up to (around) growth.

2.12.17. Triple sugar iron (TSI) agar

To determine the ability of an organism to attack a specific carbohydrate incorporated in a basal growth medium, with or without the production of gas, along with the determination of possible hydrogen sulphide (H₂S) production.

Bacto triple sugar iron agar

Beef extract	3 g	*Ferrous sulphate	0.2 g
Yeast extract	3 g	*Sodium thiosulphate (Na ₂ S ₂ O ₃).	0.3 g
Peptone	20 g	NaCl	5 g
Glucose	1 g	Phenol red	0.02 g
Lactose	10 g	Bacto agar	12 g
Sucrose	10 g	Dist. H ₂ O	1000 ml

Final pH 7.4 at 25°C

*H₂S indicator

pH Indicator (Phenol red):

Acid; yellow

Alkaline; red

Uninoculated medium; reddish-orange

Procedure:

- 1) Rehydrate with distilled water and heat gently into solution to dissolve completely.
- 2) Dispense approximately 5 ml per tube and sterilize in the autoclave for 15 minutes at 121°C.
- 3) Cool in a slanted position with deep butts.
- 4) Use a heavy inoculum, with growth from a solid culture medium.
- 5) Pick the centre of a colony with an inoculating needle. Inoculate the slant and stab the butt. Incubate for 18-24 hours.

Interpretation:

A. Carbohydrate utilization.

- 1) Glucose only fermented; peptones utilized.
Slant: alkaline (Alk), red.
Butt: acid (A), yellow.
- 2) Glucose fermented; lactose and/or sucrose fermented.
Slant/butt: A/A
- 3) Neither glucose, lactose nor sucrose fermented; peptones utilized. Slant/butt: Alk/Alk or NC/NC (i.e. only growth is evident in slant and butt and there is no change [NC] in colour, same as the uninoculated tube).

B. Gas production.

- 1) Aerogenic; CO₂ and H₂ production evident by splitting of the medium.
- 2) Anaerogenic; no gas production.

C. Hydrogen sulphide production; the presence of a black precipitate (ferrous sulphide) is evident by:

- 1) A black colour spread throughout the entire butt masking the acidity. There may even be a slight evidence on the slant.
- 2) A black ring near the top of the butt area.
- 3) A black precipitate scattered throughout the butt, not entirely masking the acidity present.

2.12.18. Urease activity

To determine the ability of an organism to hydrolyze urea, forming two molecules of ammonia by the action of the enzyme urease, with resulting alkalinity.

Bacto urea agar base

Peptone	1 g
Glucose	1 g
NaCl	5 g
Monopotassium phosphate	2 g
Urea (Difco)	20 g
Phenol red	0.01 g

Final pH 6.8 at 25°C

pH Indicator (Phenol red):

Acid; yellow (pH 6.8)
Alkaline; pinkish-red (pH 8.4)
Uninoculated medium; yellow to buff (pH 6.8)

Procedure:

- 1) Rehydrate in 100 ml distilled water and mix thoroughly to dissolve completely.
- 2) Filter sterilize the concentrated base (do not boil or autoclave).
- 3) Dissolve 15 g of Bacto agar in 900 ml distilled water by boiling and sterilize in the autoclave for 15 minutes at 121°C.
- 4) Allow to cool to 50-55°C and aseptically add 100 ml of the filter sterilized concentrated base to the cooled Bacto agar.
- 5) Mix thoroughly and dispense approximately 5 ml per tube (long slant, short butt).
- 6) Inoculate slants by spreading growth ('fish-tail') from an agar culture over entire surface of slant. Do not stab the butt (it serves as a colour control).
- 7) Incubate between 6-24 hours and every day thereafter for 6 days (longer periods may be necessary). To eliminate possible protein hydrolysis, run a control using the same test medium without urea.

Interpretation:

- A. Positive; intense pink-red (red-violet) colour on the slant. Colour may penetrate into the agar. The extent of colour indicates rate of urea hydrolysis.
- B. Negative; no colour change (buff to pale-yellow).

2.12.19. Voges Proskauer (VP)

To determine the ability of some organisms to produce a neutral end product (acetoin) from glucose fermentation.

Polypeptone	.....	7 g
Glucose	.....	5 g
Dipotassium phosphate	....	5 g
Dist. H ₂ O	.....	1000 ml

Final pH 6.9 at 25°C

Barritt VP reagent

Reagent A:	α-Naphthol	.....	5 g
	Ethyl alcohol (95%)	.....	100 ml
Reagent B:	Potassium hydroxide	.....	40 g
	Dist. H ₂ O	.....	100 ml

Procedure:

- 1) Rehydrate with distilled water and heat gently into solution.
- 2) Dispense approximately 5 ml per tube, sterilize in the autoclave for 15 minutes at 121°C and cool.
- 3) Inoculate and incubate for 24-48 hours (may require longer incubations, up to 10 days).
- 4) Add Barritt VP reagent directly to an incubated aliquot (0.6 ml of reagent A followed by 0.2 ml of reagent B).
- 5) Shake tubes gently for 1 minute to expose the medium to atmospheric oxygen in order to oxidize the acetoin, so as to obtain a colour reaction.
- 6) Allow tubes to stand at least 10-15 minutes before examination.

Interpretation:

- A. Positive; pinkish-red colour at the surface of the medium (acetoin present).
- B. Negative; yellow colour at the surface (same colour as the reagent). A copper-like colour may form, but it is still a negative reaction (due to action of the reagents when mixed).

2.13. Appendix :

Gas chromatographic methods

Figure 9. glc traces of internal and terminal epoxyalkanes.

Figure 10. glc trace of 1,2-epoxyoctane.

Solvent (S): ether

A 1-hexadecene (2 mM)

B 1-heptadecene (0.25 mM)

C 1,2-epoxyhexadecane (0.5 mM)

3% SE-30 on Chromosorb W-HP

(2.1 x 4 mm id.)

180°C isothermal

N₂ at 40 ml/min.

Figure 11. glc trace of 1,2-epoxyhexadecane.

Figure 12. glc trace of cyclopentene oxide.

Solvent (S): ether

A cyclohexene (5 mM)
B 1-nonene (0.5 mM)
C cyclohexene oxide (0.5 mM)

5% OV17 on Chromosorb W-HP
(1.5m x 4 mm id.)
40°C (5 min.) 16°C/min. to 100°C
N₂ at 40 ml/min.

Figure 13. glc trace of cyclohexene oxide.

Solvent (S): ether

A styrene (2 mM)

B styrene oxide (0.5 mM)

C 1-heptadecene (0.5 mM)

10% Carbowax 20M on Chromosorb W-HP
(1.8 x 4 mm id.)
160°C isothermal
N₂ at 40 ml/min.

Figure 14. glc trace of styrene oxide.

Solvent (S): ether

A phenyl allyl ether (2 mM)

B 1-heptadecene (1 mM)

C phenyl glycidyl ether (1 mM)

10% Carbowax 20M on Chromosorb W-HP
(1.8 x 4 mm id.)
190°C isothermal
N₂ at 40 ml/min.

Figure 15. glc trace of phenyl glycidyl ether.

Solvent (S): ether

A thioanisole (3.25 mM)

B 1-tetradecene (0.5 mM)

C phenyl methyl sulfoxide (0.5 mM)

5% OV17 on Chromosorb W-HP

(1.5 x 4 mm id.)

170°C isothermal

N₂ at 40 ml/min.

Figure 16. glc trace of phenyl methyl sulfoxide.

Solvent (S): ether

A 1-trifluoromethyl, 1-(4' ethoxy phenyl)-ethene (1mM)
 B 1-trifluoromethyl, 1-(4' ethoxy phenyl)-ethyl oxirane (0.1 mM)
 C 1-hexadecene (0.1 mM)

5% OV17 on Chromosorb W-HP
 (1.5 x 4 mm id.)
 140°C isothermal
 N₂ at 40 ml/min.

Figure 17. glc trace of 1-trifluoromethyl, 1-(4' ethoxy phenyl)-ethyl oxirane.

CHAPTER 3

RESULTS & DISCUSSION

3.1. Isolation & Characterization of alkene-utilizing bacteria

3.1.1. Isolation strategy

The ultimate objective of this project was to obtain a limited number of biocatalysts, with a broad substrate specificity, as potential epoxide producers in a biotechnological process. Chiral epoxides can be used as synthons in the production of various optically active compounds. Synthesis of non-functionalized chiral epoxides of high enantiomeric excess remains a difficult task for the organic chemist, with the notable exception of asymmetric epoxidation of allylic alcohols (Katsuki & Sharpless, 1980) and the formation of chiral 1,2-epoxypropane (Golding, 1985).

Microbial systems, on the other hand, have the advantage of exhibiting a considerable degree of stereoselectivity due to stereospecificity of the enzymes involved. Alkene- and alkane-utilizing bacteria have often been used as biocatalysts to produce optically active epoxides from gaseous olefins (Furuhashi *et al.*, 1981; Habets-Crützen *et al.*, 1985; Weijers *et al.*, 1988a), from higher olefins (Ohta & Tetsukawa, 1979; de Smet *et al.*, 1981a & 1983), from aldrin (Ferguson & Korte, 1977) and from aryl allyl ethers (Furuhashi & Takagi, 1985; Johnstone *et al.*, 1986).

Alkene-utilizing bacteria are preferred to alkane-utilizers because they: i) form epoxides in high enantiomeric excess (Habets-Crützen *et al.*, 1985), whereas the latter tend to form racemic epoxyalkanes (Subramanian, 1986; Weijers *et al.*, 1988a), ii) can only epoxidize and do not hydroxylate olefins (Habets-Crützen *et al.*, 1984) as some alkane-utilizers do (van der Linden, 1963; May & Abbott, 1973; Colby *et al.*, 1977; Hou *et al.*, 1983;

Patel *et al.*, 1983) and iii) are less sensitive to epoxyalkanes than alkane-utilizers (Habets-Crützen & de Bont, 1985).

It had been envisaged that the initial studies might involve obtaining and testing a number of alkene-utilizing bacteria known to produce chiral epoxides. However, due to the unavailability of these organisms, an extensive isolation programme was carried out using selective culture techniques. Two approaches were undertaken: i) screening for the ability to grow on 'simple' (ethene, propene) olefins on the premise that the enzyme(s) might not be highly substrate specific and ii) screening for organisms that grow on a series of 'blocked' (table 6) olefins. This latter approach was based upon the observation that although primary aliphatic carbons are more readily attacked (in nature) than olefins (Klug & Markovetz, 1971), steric hindrance (e.g. with secondary and tertiary carbons) might reverse this preference, making the attack at the double bond more likely.

Over 100 bacterial strains (mesophilic, thermophilic), covering a wide range of genera, were subsequently isolated in pure culture with gaseous or liquid (internal & terminal) olefins as the sole source of carbon and energy. Isolation and identification of the newly isolated genera are discussed in the following section.

Gaseous olefins

Ethene	
Propene	

Liquid olefins

Cyclohexene	
3,3-Dimethyl 1-butene	
4,4-Dimethyl 1-pentene	
2,3-Dimethyl 2-butene	
Allyl cyclopentane	

Table 6. Gaseous and liquid olefins as isolation substrates for alkene-utilizing bacteria.

3.1.2. Selective culture techniques

The basic stages in selective isolation of any organism involve sampling, growth on a suitable medium, incubation, colony selection and purification. Selectivity may be introduced intentionally or unintentionally at any stage.

Alkene-utilizing microorganisms were isolated from soil, mud and water (e.g. woodland, ditches, agricultural sites, downstream of chemical plants or oil refineries) in various parts of the UK. Soil samples were sieved to remove debris and larger particles. All incubations were done at 30, 45 and 65°C.

3.1.2.1. *Direct plating*

Approximately 1 g of pre-treated soil was added to 1 ml of basal mineral-salts medium (BMS). The suspension was serially diluted in the same medium and spread (0.1 ml of dilution) on dried BMS agar.

Dilute inocula (sewage, water) were, however, treated in one of the following ways: i) passed through a membrane filter (0.22-0.45 μm pore size) and placed on BMS agar, or ii) concentrated by centrifugation (13,000 g for 15 min.) and serially diluted before plating. This proved most successful, especially when the bacterial count was low.

All plates were incubated in desiccators (or in polythene containers) with gaseous (5-10% V/V) or liquid (1% V/V) olefins as substrates. Gases were removed from a football bladder and injected, whereas liquid (volatile) substrates were provided in the vapour phase by direct addition to a Bijou bottle and placing in the desiccators.

3.1.2.2. *Enrichment*

Approximately 1 g of inoculum (soil, mud) was added to 25 ml of basal liquid medium in 250 ml flasks (narrow-necked, Quick fit) sealed with Suba-seal caps (Freeman & Co., Barnsley, England). Gaseous olefins (5-10% V/V) were injected into each flask. Volatile substrates (1% V/V, filter sterilized) were added directly to the liquid medium or were provided in the vapour phase by direct addition to the centre wells of Erlenmeyer flasks, to

avoid direct contact with the organisms. The overstanding liquid was turbid after 5-7 weeks of stationary incubation. Turbidity was taken as presumptive evidence of growth of alkene-utilizers. Plates of basal medium were spread with serial dilutions of material taken from each flask and incubated in desiccators with appropriate hydrocarbon substrates. Pre-dried plates were always used because spreading a dilute suspension onto the surface of a 'wet' agar plate frequently gave rise to clumped or not so well-separated colonies. The advantage of plating onto agar medium as soon as growth appears in the first enrichment culture is to allow a number of different types of alkene oxidizers to be isolated.

Plates were examined for colony formation at 4 day intervals over a period of 3-5 weeks. This step was probably the most significant in isolation because of problems encountered during growth:

i) Presence of non alkene-utilizers that formed colonies on the basal medium by scavenging traces of organic nutrient. This was partially overcome by the use of purified Difco agar that lacked nutrients and mineral impurities. Normally, these colonies reached a maximum size in 4 days, while alkene oxidizers often did not appear until after this time. Much time and effort was, therefore, saved by picking only the late appearing colonies that increased in size over a longer incubation period.

ii) Fungal growth; the high humidity that had developed in desiccators upon prolonged incubations (3-5 weeks) stimulated fungal growth in the unsterile atmosphere of containers and on agar media. Cultures incubated in this state were often contaminated with fungi. To avoid this, a) desiccators were regularly cleaned with ethanol and contained silica gel to reduce the moisture content and b) duplicate sets of cultures were kept.

3.1.3. Culture purity and maintenance of isolates

After approximately 3-5 weeks of incubation, colonies that had developed on the dilution plates (containing between 30-300 colonies) were picked and streaked onto basal medium, solidified with purified agar, and incubated for a further 2-3 weeks with hydrocarbon substrates as the sole source of carbon and energy. Single colonies were picked

and restreaked onto fresh BMS agar *and* nutrient agar plates to ensure the absence of any scavengers that could co-exist with alkene-utilizers.

Sealed BMS plates without hydrocarbons served as controls and were incubated separately. Any subsequent growth would, therefore, be as a result of i) residual organic matter in the agar, ii) organic matter introduced with the inoculum or iii) presence of non-genuine alkene oxidizers. When a particular species developed on the control plates, it was then discounted unless a greater than 10 fold stimulation in its growth on substrate plates was evident. Isolates were considered to be pure if colonies had a similar appearance and consisted of morphologically similar organisms. In addition, each isolate was examined with a low-power objective and in a wet mount before preparing a stock culture.

Pure cultures of alkene-utilizing organisms were kept on substrate plates at 4°C and subcultured regularly (2-3 weeks). Longer term maintenance was done on nutrient agar slopes at 4°C or by storage in glycerol (10% W/V) at – 40°C.

3.1.4. Taxonomic and biochemical characterization

Pure cultures of an unidentified stock of mesophilic and thermophilic alkene-utilizing organisms, on all seven isolation substrates (table 6), were selected on the basis of colour, colonial morphology and the ease of growth on olefin plates. Initial microscopic examinations indicated the existence of different types of organisms and were, therefore, divided into three broad groups depending on shape and Gram reaction. *Group (I)* consisted of mycelial-type structures that had either surface growth or were embedded into the agar medium (tables 7 & 8). *Group (II)* included Gram-positive cocci (table 9) and *Group (III)* encompassed a variety of Gram-negative and Gram-variable rods/coccobacilli (tables 10, 11 & 11.1).

Organisms from each group were subsequently identified according to Bergey's Manual of Systematic Bacteriology (vol. I, 1984; vol. II, 1986).

Table 7. Taxonomic and biochemical properties of olefin-utilizing *Streptomyces* spp.

Isolate No.	M 119 C	M 120 D	M 121 C	M 129 C	M 131 A	M 146	M 153 C	M 155 A
Isolation method	Direct	Direct	Direct	Direct	Direct	Filtration	Filtration	Direct
Isolation substrate	Cyclohexene	Cyclohexene	Cyclohexene	2,3-DMB	2,3-DMB	4,4-DMP	3,3-DMB	3,3-DMB
Isolation temp. °C	45	30	30	30	30	30	30	30
Colonial morphology	Embedded 2-3 mm Cream Entire Convex	Embedded 2-3 mm Cream Entire Convex	Embedded 2 mm Cream Entire Convex	Embedded 2-3 mm Cream Entire Convex	Embedded 2-3 mm Cream Entire Convex	Embedded 0.5 mm Cream Entire Convex	Embedded <0.1 mm No pigment Entire Convex	Embedded <0.1 mm Cream Entire Convex
Acid-fast	–	–	–	–	–	–	–	–
Catalase	–	–	–	Weak	–	+	–	+
Gram stain	Variable	+	Variable	Variable	Variable	Variable	Variable	+
Motility	–	–	–	–	–	–	–	–
Nitrate reduction	+	+	–	Weak	–	–	Weak	+
Oxidase	–	–	–	–	–	–	–	–
Simmons citrate	–	–	–	–	+	–	–	–

Morphological description is as a result of growth on Difco nutrient agar (NA) except otherwise stated.

Slide culture of each organism showed mycelial-type structures with aerial hyphae that did not fragment. Septa formation was also evident.

2,3-DMB   2,3-dimethyl 2-butene
 4,4-DMP   4,4-dimethyl 1-pentene
 3,3-DMB   3,3-dimethyl 1-butene

3.1.4.1.

*Group (I): Mycelial-type***Table 8. Taxonomic and biochemical properties of olefin-utilizing *Nocardia* spp.**

Isolate No.	M 140	M 150 C	M 159 B	M 173 A
Isolation method	Direct	Enrichment	Enrichment	Enrichment
Isolation substrate	3,3-DMB	3,3-DMB	3,3-DMB	4,4-DMP
Isolation temp. °C	30	30	45	30
Colonial morphology	Mucoid 3-4 mm Cream Irregular Flat	Mucoid >1 mm Brown Entire Convex	Mucoid <0.5 mm White Entire Pin-pointed	Mucoid >2 mm Cream Entire Convex
Acid-fast	Partial	Partial	Partial	Partial
Catalase	+	+	+	+
Gram stain	Variable	Variable	Variable	Variable
Motility	–	–	–	–
Nitrate reduction	+	+	–	+
Oxidase	+	–	–	+
Simmons citrate	+	+	–	+

Morphological description is as a result of growth on Difco nutrient agar (NA) except otherwise stated.

Slide culture of each organism showed extensive mycelial forms. Primary branching was evident in young cultures which easily fragmented in older cultures to coccoid and bacillary forms. Growth occurred on the surface of agar.

3,3-DMB   3,3-dimethyl 1-butene
4,4-DMP   4,4-dimethyl 1-pentene

3.1.4.2.

*Group (II): Coccoid***Table 9. Identification of Gram-positive olefin-utilizing bacteria.**

Isolate No.	M 90 C	M 97 B	M 150 G	M 175 C	M 176 A	M 186
Isolation method	Enrichment	Enrichment	Enrichment	Enrichment	Enrichment	Enrichment
Isolation Substrate	Ethene	Ethene	3,3-DMB	ACP	ACP	Ethene
Isolation temp. °C	30	30	30	30	30	30
Colonial morphology	Smooth 1-2 mm on MS plates Yellow Entire Convex	Sticky Yellow	Mucoidal >1 mm Orange Entire Convex	Mucoidal 3-4 mm Yellow Entire Convex	Mucoidal >1 mm No pigment Entire Convex	Mucoidal 0.1-0.5 mm Yellow Entire Convex
Cellular morphology	Coccus In pairs Scattered	Coccus 2-4 in chains	Coccus In pairs Scattered	Coccus 2-3 in chains	Coccus 2-4 in chains	Coccus 3-4 in chains
Arginine hydrolysis	+	+	+	-	-	-
Catalase	Weak	Weak	+	+	+	Weak
CF	Inert	Fermentative	Inert	Inert	Inert	Fermentative
Esculin hydrolysis	-	-	-	-	-	+
Gelatin liquefaction	+	-	-	+	-	-
Motility	-	-	-	-	-	-
NA plus 5-10% NaCl	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	-
OF-glucose	Inert	Inert	Inert	Inert	Inert	Fermentative
OF-lactose	Inert	F (G)	Inert	Inert	Inert	Fermentative
Oxidase	+	-	+	+	+	-
Simmons citrate	-	-	-	-	-	-
Starch hydrolysis	-	-	-	-	-	-
Urease	-	+	-	-	-	-
Voges Proskauer	-	+	-	+	-	-
Genus	<i>Micrococcus</i> sp.	<i>Staphylococcus</i> sp.	<i>Micrococcus</i> sp.	<i>Micrococcus</i> sp.	<i>Micrococcus</i> sp.	<i>Aerococcus</i> sp.

Morphological description is as a result of growth on Difco nutrient agar (NA) except otherwise stated.

Inert metabolism indicates that the organism does not produce enough acid in the medium to change the colour of indicator in OF-tubes. Carbohydrates used were glucose, lactose, mannitol, sucrose or xylose.

3,3-DMB	3,3-dimethyl 1-butene
ACP	allyl cyclopentane
OF	oxidation fermentation test
F	fermentative metabolism
(G)	gas production
MS	mineral-salts
CF	carbohydrate fermentation

3.1.4.3.

*Group (III): Rods & Coccobacilli***Table 10. Identification of ethene-utilizing bacteria.**

Isolate No.	M 26	M 51	M 79 B	M 88 A	M 93 A	M 100	M 101	M 182 B	M 184
Isolation method	Enrichment	Enrichment	Enrichment	Direct	Enrichment	Filtration	Enrichment	Direct	Direct
Isolation substrate	Ethene	Ethene	Ethene	Ethene	Ethene	Ethene	Ethene	Ethene	Ethene
Isolation temp. °C	30	30	30	30	30	30	30	65	30
Colonial morphology	Slimy Yellow	Mucoidal 1 mm Cream Entire Convex	Mucoidal 2 mm Cream Irregular Convex	Mucoidal 2-6 mm No pigment Irregular Flat	Mucoidal 2-5 mm Orange Entire Convex	Mucoidal 1 mm No pigment Entire Convex	Mucoidal 1-2 mm No pigment Entire Convex	Mucoidal 1 mm Cream Entire Convex	Mucoidal 2-3 mm Orange Irregular Flat
Cellular morphology	Coccobacilli Uniform Scattered	Rods Pleomorphic Scattered	Rods Pleomorphic 2-3 in chains	Coccobacilli Uniform Scattered	Coccobacilli Uniform Few in chain	Coccobacilli Uniform Scattered	Coccobacilli Uniform Few in chain	Rods Uniform Scattered	Rods Uniform Few in chain
Catalase	–	–	+	–	–	+	+	–	+
Esculin hydrolysis	–	–	–	–	+	+	–	–	–
Fluorescein	–	–	–	–	–	–	–	–	–
Gelatin liquefaction	+	+	–	+	+	+	+	+	+
Gram stain	Variable	–	–	Variable	–	–	–	–	–
Indole test	–	–	–	–	–	–	–	–	–
MacConkey agar	LF+	–	Diffuse NLF+	–	–	NLF+	NLF+	–	NLF+
Methyl red	–	–	–	–	–	–	–	–	–
Motility	–	–	–	+	–	+	+	+	+
Nitrate reduction	+	+	–	–	–	+	+	–	+
OF-glucose	Inert	Inert	Inert	Inert	Inert	Fermentative	Inert	Inert	Inert
Oxidase	–	+	+	+	–	+	+	+	+
Simmons citrate	–	–	–	–	–	+	+	–	–
Starch hydrolysis	–	–	–	–	–	–	–	–	–
Triple sugar iron	NC/NC	NC/NC	AIK/NC*	AIK/NC	NC/NC	AIK/NC	AIK/NC	NC/NC	NC/NC
Urease	–	+	–	–	–	–	+	+	+
Genus	<i>GDG</i>	<i>GDG</i>	<i>Moraxella</i> sp.	<i>GDG</i>	<i>GDG</i>	<i>Aeromonas</i> sp.	<i>Alcaligenes</i> sp.	<i>GDG</i>	<i>Pseudomonas</i> sp.

Morphological description is as a result of growth on Difco nutrient agar (NA) except otherwise stated.

GDG group-difficult genera are those with unusual biochemical properties that do not readily fall into the genera described in Bergey's Manual of Systematic Bacteriology.

NLF non-lactose fermentor
LF lactose fermentor
AIK/NC slant: alkaline/butt: no change
NC/NC no change in slant or butt
* H₂S production

3.1.4.3.

*Group (III): Rods & Coccobacilli***Table 11. Identification of Gram-negative olefin-utilizing bacteria.**

Isolate No.	M 189 K	M 189 L	M 190 A	M190 B	M 147 A	M 148	M 159C
Isolation method	Enrichment	Direct	Enrichment	Filtration	Enrichment	Enrichment	Enrichment
Isolation Substrate	Propene	Propene	Propene	Propene	3,3-DMB	3,3-DMB	3,3-DMB
Isolation temp. °C	30	30	30	30	30	30	45
Colonial morphology	Mucoidal 1-2 mm Yellow Entire Convex	Mucoidal 1 mm Cream Entire Convex	Mucoidal 2 mm Cream Entire Convex	Mucoidal <1 mm Cream Entire Convex	Mucoidal 2-5 mm Yellow Irregular Flat	Mucoidal 1-2 mm Cream Entire Convex	Mucoidal 2 mm Cream Entire Flat
Cellular morphology	Rods Uniform Scattered	Coccobacilli Uniform In pairs	Rods Uniform In chains	Rods Uniform 2-3 in chains	Long rods Uniform Scattered	Rods Uniform Scattered	Rods Uniform Scattered
Catalase	+	Weak	+	-	-	+	-
Esculin hydrolysis	+	+	+	-	-	+	-
Fluorescein	-	-	-	-	-	-	-
Gelatin liquefaction	+	+	+	+	+	+	+
Indole test	-	-	-	-	-	-	-
MacConkey agar	NLF+	-	NLF+	-	-	Diffuse NLF+	-
Methyl red	-	-	-	-	-	-	-
Motility	-	-	-	-	+	+	+
Nitrate reduction	+	-	+	+	+	+	-
OF-glucose	Fermentative	Fermentative	Inert	Inert	Inert	Fermentative	Inert
Oxidase	-	+	+	-	+	+	+
Simmons citrate	+	-	+	-	-	+	-
Starch hydrolysis	+	-	-	-	-	+	-
Triple sugar iron	A/A	A/NC	AIK/NC	NC/NC	NC/NC	A/A	AIK/NC
Urease	+	-	+	-	-	+	-
Genus	<i>GDG</i>	<i>GDG</i>	<i>GDG</i>	<i>GDG</i>	<i>GDG</i>	<i>Vibrio</i> sp.	<i>GDG</i>

Morphological description is as a result of growth on Difco nutrient agar (NA) except otherwise stated.

GDG group-difficult genera are those with unusual biochemical properties that do not readily fall into the genera described in Bergey's Manual of Systematic Bacteriology.

3,3-DMB 3,3-dimethyl 1-butene
 NLF non-lactose fermentor
 AIK/NC slant: alkaline/butt: no change
 NC/NC no change in slant or butt
 A/A acidic slant and butt
 A/NC slant: acidic/butt: no change

3.1.4.3.

Group (III): Rods & Coccobacilli**Table 11.1. Identification of Gram-negative olefin-utilizing bacteria.**

Isolate No.	M 167 A	M 170 H	M 172 A	M 173 C	M 176 C	M 177 E
Isolation method	Enrichment	Enrichment	Enrichment	Enrichment	Enrichment	Enrichment
Isolation Substrate	4,4-DMP	4,4-DMP	4,4-DMP	4,4-DMP	ACP	ACP
Isolation temp. °C	30	30	30	30	30	45
Colonial morphology	Mucoidal 1 mm Cream Irregular Flat	Mucoidal 1-2 mm Yellow Entire Convex	Mucoidal 2-5 mm Green Irregular Flat	Mucoidal 2-3 mm Pale green Entire Convex	Mucoidal 1-2 mm No pigment Entire Convex	Mucoidal 1-2 mm Cream Entire Flat
Cellular morphology	Thin rods Uniform Scattered	Rods Pleomorphic Scattered	Thin rods Uniform Scattered	Thin rods Uniform Scattered	Rods Uniform In chains	Thin rods Uniform Scattered
Catalase	+	+	+	+	+	–
Esculin hydrolysis	+	+	–	–	–	–
Fluorescein	–	–	–	–	–	–
Gelatin liquefaction	+	+	+	–	+	+
Indole test	–	–	–	–	–	–
MacConkey agar	Diffuse NLF+	NLF+	NLF+	Diffuse NLF+	NLF+	–
Methyl red	–	–	–	–	–	–
Motility	–	+	+	–	+	+
Nitrate reduction	+	+	Weak	+	+	–
OF-glucose	Inert	Inert	Oxidative	Oxidative	Inert	Inert
Oxidase	+	+	+	+	+	+
Simmons citrate	+	+	+	+	+	–
Starch hydrolysis	–	–	+	–	–	–
Triple sugar iron	AIK/NC	AIK/NC	NC/NC	NC/NC	AIK/NC	AIK/NC
Urease	+	–	+	+	+	–
Genus	GDG	<i>Pseudomonas</i> sp.	<i>Pseudomonas</i> sp.	<i>Flavobacterium</i> sp.	<i>Alcaligenes</i> sp.	GDG

Morphological description is as a result of growth on Difco nutrient agar (NA) except otherwise stated.

GDG group-difficult genera are those with unusual biochemical properties that do not readily fall into the genera described in Bergey's Manual of Systematic Bacteriology

4,4-DMP 4,4-dimethyl 1-pentene

ACP allyl cyclopentane

NLF non-lactose fermentor

AIK/NC slant: alkaline/butt: no change

NC/NC no change in slant or butt

3.1.5. Selection of suitable bacteria

The newly isolated organisms, on all seven substrates, were initially screened for the ability to grow in *liquid* basal mineral-salts medium with gaseous or liquid olefins as the sole source of carbon and energy.

3.1.5.1. Gaseous olefins

Mesophilic ethene- and propene-utilizing bacteria were cultivated in batch 100 ml Erlenmeyer flasks containing 20 ml of a sterile BMS medium with 5-10% (V/V) gaseous olefins. The inoculum consisted of cells pre-adapted on each substrate. Organisms were streaked onto mineral-salts plates and incubated at 30°C for ten days in an olefin/air atmosphere. Single colonies that had developed were used as inocula for seed flasks.

Flasks with no substrate served as controls. Cell growth, as evident by culture turbidity (sometimes accompanied by pellicle growth), was determined during four weeks of incubation (Lh fermentation^{*}, 300 rpm). Eighteen organisms were, therefore, selected on the basis of growth rate on ethene or propene.

*rotary shaker

3.1.5.2. Liquid olefins

Liquid (internal & terminal) alkene-utilizing bacteria included numerous genera: *Alcaligenes*, *Flavobacterium*, *Micrococcus*, *Nocardia*, *Pseudomonas*, *Streptomyces* and *Vibrio* spp.

Each substrate (filter sterilized) was tested at various concentrations (0.1-10% V/V) by direct addition to sterile basal medium or to the centre wells of Erlenmeyer flasks (250 ml containing 50 ml of medium) and incubated (30 or 45°C) statically, or shaking for up to a period of one month. Cells were pre-adapted on olefin plates prior to inoculation. None of the isolates, however, grew in liquid BMS despite growth on substrate plates. Medium composition was modified at this stage to include additional trace elements and co-factors. The new medium had the following composition (per litre of dist. H₂O):

NH₄Cl (2 g); CaCl₂·2H₂O (2.5 mg); MgSO₄·7H₂O (0.27 g); MnCl₂·4H₂O (3 mg), FeCl₃·6H₂O (1 mg); ZnSO₄·7H₂O (0.8 mg); CuSO₄·5H₂O (0.4 mg); CoCl₂·6H₂O (0.1 mg); NiCl₂·6H₂O (0.2 mg); H₃BO₃ (0.02 mg); EDTA (0.5 mg); Biotin (100 µg); Thiamin (100 µg); Bacto agar (16 g) for solid medium. Sterile phosphate buffer (pH 7) was added after autoclaving to a final concentration of 25 mM [100 ml/l of K₂HPO₄ (44 g); KH₂PO₄ (34 g) stock solution].

Similarly, it was not possible to observe growth in liquid medium, despite numerous procedural modifications (different concentrations of olefins, temperature, direct/indirect substrate addition). All of the organisms, however, grew well on both salts media supplemented with 0.5% (W/V) glucose or succinate, 0.01% (W/V) yeast extract or in nutrient broth, but failed to grow when subcultured into a fresh basal medium. Cultivation with gaseous olefins (ethene, propene, 1-butene) resulted in very poor or no growth, even after 6 weeks of incubation.

3.1.5.3. Thermophilic bacteria

The temperature profile (30-65°C) of a number of unidentified thermophilic (65°C) gaseous and liquid alkene-utilizing bacteria on salts/nutrient agar plates showed that growth was: i) fast and abundant at 55-65°C, ii) slow at 37-45°C and iii) non-existent at 30°C. Similar problems were encountered for growth in liquid in either salts medium.

The concentration of hydrocarbons in aqueous solution coupled with the ability to metabolize such compounds, are the main factors determining their toxicity. Various substrate concentrations (0.1-10% V/V) were used to establish growth. From the solubility data (tables 1 & 2) it became evident that the olefins, in general, had enough solubility to support cellular growth. To see whether toxicity might account for the lack of growth, 5-10% (V/V) pristane (2,6,10,14-tetramethyl pentadecane) was included in the basal medium with olefins as the sole source of carbon and energy. Pristane is a biologically inert hydrocarbon that is not metabolized by these organisms. Addition of a non-toxic compound

with which the substrate is miscible should relieve toxic effects by decreasing its concentration in the aqueous phase (Gill & Ratledge, 1972). None of the isolates, however, grew in either salts medium, even in the presence of pristane.

Benson *et al.*, (1979) have proposed a model in which alkane molecules spontaneously partition into the interior of the bilayer membrane, without intervention of a specific transport system. If this were the case, then impaired transport of the substrate would probably not be responsible for the lack of growth.

The most widely used method to test the ability of an organism to form epoxides, involves cultivation in a suitable liquid medium, allowing optimum growth, followed by harvesting and resuspension of the biomass in a simple 'transformation medium' (e.g. a buffer solution with optimized pH value containing the substrate) where bioconversion is carried out. The strict separation of microbial growth and biotransformation offers the following advantages: i) each step can individually be optimized and ii) * can be conducted under non-sterile conditions. An alternative method for obtaining biomass may involve cultivation on large plates prior to bioconversion (given the inability of some of the newly isolated alkene-utilizers to grow in liquid medium despite growth on substrate plates). Economic and practical considerations may, however, limit its application for commercial use.

Screening for the ability to grow in liquid medium resulted in the selection of 18 ethene- and propene-utilizing bacteria. The organisms included *Aerococcus*, *Alcaligenes*, *Micrococcus* and *Staphylococcus* spp. and a number of Gram-negative, Gram-positive and Gram-variable mesophilic rods/coccobacilli not yet identified. Basal mineral-salts medium (without the additional trace elements and co-factors) was used for cultivation in all subsequent experiments. A *Micrococcus* sp. M90C was initially selected to investigate its potential as a 'general-purpose' biocatalyst for production of chiral epoxides. The selection criteria were: i) growth rate on substrate of isolation (ethene), ii) ability to utilize olefins (C₂-C₄) but not saturated hydrocarbons and iii) preliminary evidence of epoxide accumulation (as analyzed by glc).

*biotransformation

3.2.

Micrococcus sp. M90C

3.2.1. Characterization

An ethene-utilizing organism (designated M90C) was isolated in pure culture from soil using classical enrichment techniques. The bacterium is a Gram-positive coccus, characteristically occurring in pairs (diplococcus) and immotile in 'hanging-drop' preparations. On solid BMS medium colonies appeared yellow, well-separated, 1-2 mm in diameter, slightly raised and smooth with an entire edge (fig. 18). Growth was evident within 3 days at 30°C (colonies were well formed in 6-7 days) with ethene (5% V/V) as the sole source of carbon and energy. In liquid BMS medium uniform turbidity, followed by a somewhat mucoidal deposit upon standing, was observed. Nutrient agar (with or without 5-10% W/V NaCl) supported growth; the colonies were yellow, sticky and produced slime.

Arginine and gelatin were hydrolyzed, whereas esculin and starch were not. The hydrolytic ability was determined after 3-6 days at 30°C. The ability to ferment various carbohydrates was determined by acid production in Hugh & Leifson OF-basal medium supplemented with glucose, lactose, mannitol, sucrose or xylose. Not enough acid was, however, produced to change the colour of the indicator in OF-tubes (inert metabolism). Catalase reaction was weak, citrate was utilized (Simmons citrate), nitrate was produced from nitrate broths, oxidase reaction was positive, Voges Proskauer (VP) and urease tests were, however, negative.

Strain M90C was therefore identified as a *Micrococcus* sp. on the basis of the above criteria (Bergey's Manual, 1984 & 1986).

3.2.2. Growth on various substrates

Unsaturated hydrocarbons (ethene, propene, 1-butene, 1,3-butadiene) supported growth, whereas alkanes (methane, ethane, propane, butane) or *cis-/trans*-2-butenes did not, even after prolonged incubations (4 weeks). 1,2-Epoxyalkanes (C₂-C₄) were utilized

A

B

Figure 18. Ethene-grown *Micrococcus* sp. M90C.

A) Plate

B) Phase-contrast light micrograph

after a long lag. Glucose, lactose, pyruvate, and succinate (0.5% W/V), propionate, 1-propanol and 1,2-propanediol (0.2% V/V) also supported growth.

The bacterium was able to grow well on higher concentrations of ethene (up to 50% V/V) despite its initial isolation at 5% (V/V), demonstrating its tolerance to the higher levels of olefin.

3.2.3. Epoxidation of gaseous olefins *see overleaf

Ethene-grown *Micrococcus* sp. M90C accumulated epoxyalkanes at various rates depending on the olefin used (table 12). Control experiments with heat-killed suspensions indicated that the epoxide was produced enzymatically. Reaction mixtures lacking oxygen or substrate were also incapable of product formation. Product identification was done by retention time comparisons, co-chromatography with authentic standards and acid hydrolysis (May & Abbott, 1973).

Terminal olefins (C_2 - C_4) were solely epoxidized but not hydroxylated. The terminal carbons of *trans*-2-butene were not hydroxylated either. The possibility of a transient formation of crotyl alcohol (2-butene-1-ol) that may have rapidly transformed to crotonaldehyde (or crotonic acid), was discounted as none of these compounds could be detected during the reaction (12 hours). Similarly, alkanes (propane, butane) were not hydroxylated (analysis by glc).

This is consistent with the general finding that alkene-utilizing bacteria only epoxidize olefins and do not hydroxylate alkanes or alkenes (Habets-Crützen *et al.*, 1984).

3.2.4. Enantiomeric composition of epoxyalkanes

The enantiomeric composition of microbial 1,2-epoxypropane, 1,2-epoxybutane and *trans*-2,3-epoxybutane was investigated by chiral complexation capillary glc. Analysis of the head-space of reaction vessels and comparison of relative peak areas provides a precise measurement of the chirality of volatile epoxyalkanes without chemical manipulations, isolation or purification (Schurig & Bürkle, 1982). The resolution of racemic and microbial

*Micrococcus sp. M90C was grown in batch for 6 days ($OD_{600}=0.57$) with ethene (5-10% V/V) as the sole source of carbon and energy. The bioconversion yields ($Y_{p/s}$) were based on the amount of substrate fed to the organism and were as follows: epoxyethane (52 mole%), 1,2-epoxypropane (45 mole%), 1,2-epoxybutane (96 mole%) and *trans*-2,3-epoxybutane (11 mole%).

Specific activities were calculated from the initial rates of reaction during which the product formation was linear. The units of specific activity (nmole/min./mg protein) were then converted into μ mole/hr./g. DW (i.e. specific productivity).

Table 12. Production of epoxyalkanes by *Micrococcus* sp. M90C.

Accumulated product	Specific* activity	Specific** productivity
Epoxyethane	17	235
1,2-Epoxypropane	10	139
1,2-Epoxybutane	15	208
<i>trans</i> -2,3-Epoxybutane	5	69

* nmole/min./mg protein

** μ mole/hr./g. DW.

Growth condition: 6 day cultivation, batch
5-10% (V/V) ethene.

Reaction: 25mM phosphate buffer, pH 7.0 at 30°C.
(non-optimized) Bioconversion was started by injecting 10-40% (V/V) olefins.
All incubations were done in triplicate.

Total protein 0.6 mg/ml

Dry weight (DW) 2.6 g/l

OD₆₀₀ Culture 0.57
Resting-cells 14.0

epoxides, on an optically active nickel compound (which serves as an enantioselective complexing stationary phase) is shown in figure 19.

To confirm that the two peaks observed with ($\pm$) 1,2-epoxypropane corresponded to *R*- and *S*-enantiomers and not to any possible product formed by reaction of epoxypropane with the column material, the pure isomers were injected separately onto the column. Each isomer produced only one peak in the glc trace. Identification of components in the racemic mixture was done by co-injections and retention time comparisons (fig. 20). The *S*-enantiomer is eluted later than the *R*-antipode on the nickel complex derived from ($\pm$)-1*R*-camphor (Schurig *et al.*, 1978).

Comparison with known elution patterns (Schurig *et al.*, 1978 & 1980; Schurig, 1983) and peak area integration, showed that *Micrococcus* sp. M90C formed *R*-epoxyalkanes (ee=84-96%) with a very high degree of enantioselectivity (table 13).

The chirality of microbial epoxidations might be affected by an isomerization process, in which a specific 'isomerase' could be present that isomerizes only the newly formed epoxides *before* their release into free solution (May *et al.*, 1977), causing the *R/S* ratio to change during the reaction. Periodic analysis of the head-space of the incubations, however, showed that the ratio remained constant.

3.2.5. Low temperature storage stability

Storage (25 mM phosphate buffer, pH 7) stability for production of epoxyethane by *Micrococcus* sp. M90C was investigated. All of the epoxidation activity (96-99% of the initial value) was retained on storage at -40°C over a period of six months. Activity was also fairly stable to short-term storage at +4°C, but was lost when kept on ice for up to nine days (fig. 21).

Thus, whenever possible, cells were freshly grown, otherwise frozen aliquots were thawed prior to use.

Racemic:

A *trans*-2,3-epoxybutane
 B 1,2-epoxypropane
 C 1,2-epoxybutane

Micrococcus sp. M90C:

A' *trans*-(2*R*,3*R*)-epoxybutane
 B' *R*-1,2-epoxypropane
 C' *R*-1,2-epoxybutane

Figure 19. Enantiomeric resolution of epoxyalkanes by chiral complexation capillary glc.

Figure 20. Resolution of racemic 1,2-epoxypropane by chiral complexation capillary glc.

Table 13. Enantiomeric composition of epoxyalkanes by ethene-grown *Micrococcus* sp. M90C.

Accumulated product	<i>R</i>	<i>S</i>	ee
1,2-Epoxypropane	98	2	96
1,2-Epoxybutane	97	3	94
<i>trans</i> -2,3-Epoxybutane	92	8	84

Enantiomeric excess (ee) = $\frac{R-S}{R+S} (100)$

Values in relative %, (±1%) as analyzed by chiral complexation capillary glc. All incubations were done in triplicate.

Reaction:	25mM phosphate buffer, pH 7.0 at 30°C. Bioconversion was started by injecting 10-40% (V/V) olefins.
Total protein	0.6 mg/ml
Dry weight	2.6 g/l
OD ₆₀₀ Culture	0.57
Resting-cells	14.0

Figure 21. Storage stability for production of epoxyethane by *Micrococcus* sp. M90C.

Growth condition:	6 day cultivation, batch 5-10% (V/V) ethene.
Reaction: (non-optimized)	25 mM phosphate buffer, pH 7.0 at 30°C. Bioconversion was started by injecting 10-40% (V/V) ethene. All incubations were done in triplicate.
Maximal activity	17.0 nmole/min./mg protein
Total protein	0.6 mg/ml
Dry weight	2.6 g/l
OD ₆₀₀ Culture	0.57
Resting-cells	14.0

3.2.6. Process optimization

Production of epoxyethane by *Micrococcus* sp. M90C was chosen as a model system to optimize pH, temperature, buffer and substrate concentration.

3.2.6.1. pH Optimum

Ethene-grown cells (1.3 g/l DW) were resuspended in seven buffer solutions (25 mM, pH 6-12): citric acid/ Na_2HPO_4 , pH 6-7.5; $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 6-8; tris/Cl, pH 7.5-8.5; glycine/NaOH, pH 8.6-10.5; $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, pH 9-10.8; borax/NaOH, pH 9.3-10.7; $\text{Na}_2\text{HPO}_4/\text{NaOH}$, pH 11-12.

The pH optimum for production of epoxyethane was at 10.5 with 25 mM glycine/NaOH buffer (fig. 22a). Similar amounts of epoxide were formed at the overlapping pH regions of each buffer. Additionally, the pH optimum was re-examined with different buffers (borax/NaOH; $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$) at the same pH region (pH 9-10.8). Production rates were similar and maximal activity (58 nmole/min./mg protein) was at pH 10.5 with all such buffers.

The buffering capacity of each solution was checked by measuring the pH before and after each bioconversion. The final pH was around the initial value (± 0.01 pH unit).

The high pH optimum (10.5) for production of epoxyethane by *Micrococcus* sp. M90C is unusual in that other alkane- or alkene-utilizing bacteria possess monooxygenases that have lower pH optima. This may therefore reflect on the differences in the genera and/or on the properties of the enzymes involved.

Figure 22. Optimization of epoxyethane production by *Micrococcus* sp. M90C.

Data was obtained from three independent experiments.
All incubations were done in triplicate.

Growth condition: 5½ day cultivation, batch
5-10% (V/V) ethene.

Total protein 0.3 mg/ml
Dry weight 1.3 g/l

OD₆₀₀ Culture 0.54
Resting-cells 6.6

Assays were carried out for 1 hour during which
the production of epoxyethane was linear.

3.2.6.2. pH Profile for production vs degradation

Micrococcus sp. M90C contains an epoxide-degrading enzyme that further oxidizes epoxyethane. Degradation was investigated by incubation (35°C) of 'resting-cells' (1.3 g/l DW) with an exogenous epoxide solution (1 mM) over the pH range 7-10.5. Hydrolysis of epoxyethane was very slow and decreased linearly with increasing pH. Degradation was minimal at the optimum pH (10.5) for production (table 14). Non-enzymatic oxidation of epoxide by heat-killed cells at each pH region, was negligible in the standard assay.

Formation of epoxyethane is dependent on the balance of two factors; enzymatic production vs degradation. The slow hydrolysis of product, therefore, allows the organism to accumulate epoxyalkanes (C₂-C₄). An accurate stoichiometry for production vs degradation cannot and should not be reliably quoted since the latter is only a measure of disappearance rate of an *exogenous* epoxide solution which may not be true under physiological conditions.

3.2.6.3. Optimization of temperature, buffer and substrate concentration

The temperature optimum for production of epoxyethane was at 35°C with 25 mM glycine/NaOH buffer, pH 10.5 (fig. 22b). Various buffer concentrations (5-100 mM) affected production rate. Activity was maximal (81 nmole/min./mg protein) with 5mM buffer; a 33% inhibition was, however, evident with 100 mM buffer (fig. 22c). Varying ethene concentrations (5-80% V/V) in the gas phase of reaction vessels, did not appreciably affect production rate. The optimum value was at 10-40% (V/V) [fig. 22d].

Process optimization has therefore resulted in a five fold increase in production of epoxyethane by *Micrococcus* sp. M90C (fig. 23). The optimized value is higher than any previous reports on alkane- or alkene-utilizing bacteria (table 15). The optimized conditions are: glycine/NaOH buffer (5 mM), pH 10.5 at 35°C with 10-40% (V/V) ethene.

*It should, however, be borne in mind that some of the bioconversion rates quoted by other authors may have not been obtained under the optimized conditions. This should therefore be taken into account for comparative purposes.

Table 14. pH Profile for degradation of epoxyethane by *Micrococcus* sp. M90C.

pH of reaction	Degradation rate*
7.0	1.4
8.0	1.1
9.0	0.95
10.0	0.7
10.5	0.5

* nmole/min./mg protein

Growth condition: 5½ day cultivation, batch
5-10% (V/V) ethene.

Reaction: 25 mM appropriate buffer at 35°C.
Bioconversion was started by adding 1 mM (final conc.) exogenous epoxyethane.
All incubations were done in triplicate.

Total protein 0.3 mg/ml
Dry weight 1.3 g/l

OD₆₀₀ Culture 0.54
Resting-cells 6.6

Figure 23. Effect of optimization on microbial production of epoxyethane.

A	25 mM	Phosphate buffer	pH 7.0	30°C
B	25 mM	Glycine buffer	pH 10.5	30°C
C	25 mM	Glycine buffer	pH 10.5	35°C
D	5 mM	Glycine buffer	pH 10.5	35°C

Table 15. A comparison of catalytic activity of various alkane- and alkene-utilizing bacteria for the epoxidation of ethene.

Organism	Growth substrate	Bioconversion rate*	Reference
<i>M. capsulatus</i>	Methane	46	Hou <i>et al.</i> (1979)
<i>M. organophilum</i>	Methane	8	Hou <i>et al.</i> (1979)
<i>M. trichosporium</i>	Methane	16	Hou <i>et al.</i> (1979)
<i>Acinetobacter</i> spp.	Propane	8	Hou <i>et al.</i> (1983)
<i>Alcaligenes</i> spp.	Propane	43	Hou <i>et al.</i> (1983)
<i>Arthrobacter</i> spp.	Butane	38	Patel <i>et al.</i> (1983)
<i>Brevibacterium</i> spp.	Propane	28	Hou <i>et al.</i> (1983)
<i>Mycobacterium</i> spp.	Butane	17	Patel <i>et al.</i> (1983)
<i>Nocardia</i> spp.	Propane	24	Hou <i>et al.</i> (1983)
<i>Pseudomonas</i> spp.	Propane	28	Hou <i>et al.</i> (1983)
<i>Nitrosomonas europaea</i>	Ammonium salts	4	Hyman & Wood (1984)
<i>Mycobacterium</i> sp. Py8	Propene	25	Habets-Crützen <i>et al.</i> (1984)
<i>Xanthobacter</i> sp. Py2	Propene	46	van Ginkel <i>et al.</i> (1986)
<i>Nocardia</i> sp. BT1	Butadiene	17	van Ginkel <i>et al.</i> (1987b)
<i>Micrococcus</i> sp. M90C	Ethene	81	This work.

* nmole/min./mg protein

3.2.7. Induction of epoxidation activity

The effect of growth substrates on formation of epoxyethane by *Micrococcus* sp. M90C was investigated. Ethene- and glucose-grown cells (1.1-1.17 g/l DW) accumulated (1-5 mM) the epoxide, and its production was not dependent on the substrate used to cultivate the organism. The ability to form epoxyethane was repressed by growth on glucose but subsequently induced by ethene (fig. 24). Cells growing on a mixture of glucose and ethene reached a higher density and had a similar bioconversion rate, to cells grown on ethene alone. Given the problems encountered with large scale growth of microorganisms on gaseous hydrocarbons, this may be a preferable alternative for production of catalytically active cells.

Similarly, *Nocardia corallina* B276 (Furuhashi *et al.*, 1981 & 1986) epoxidizes terminal olefins (C_2 - C_{18}) when grown on glucose, whereas the other organisms tested to date are only induced by growth on alkanes or alkenes (Weijers *et al.*, 1988a). *N. corallina*, however, hydroxylates olefins as well as alkanes but *Micrococcus* sp. M90C does not. The optical purity of epoxyalkanes (ee=84-96%, table 13) is also much higher than with *N. corallina* (ee=46-74%) [Weijers *et al.*, 1988a].

Epoxyethane was further metabolized by *Micrococcus* sp. M90C as observed by a very slow enzymatic hydrolysis upon prolonged incubation (fig. 24). During the formation of 1,2-epoxyalkanes by alkene-utilizing bacteria a continuous regeneration of the co-factor NADH is essential, since formation of the epoxides is brought about by a NADH-dependent monooxygenase (de Bont *et al.*, 1979 & 1983). Reduction of NAD^+ can be achieved through endogenous respiration during a limited period of time, but for prolonged epoxide formation an external NADH-regenerating co-substrate is required (de Bont *et al.*, 1983; Drozd & Bailey, 1984; Habets-Crützen & de Bont, 1987). A continued production of epoxyethane (19 hours) therefore, indicates that glycine may serve as a co-substrate for NADH-regeneration in *Micrococcus* sp. M90C.

Figure 24. Effect of growth substrates on epoxidation activity.

Reaction: 5 mM glycine/NaOH buffer, pH 10.5 at 35°C.
All incubations were done in triplicate.
Bioconversion was started by injecting
10-40% (V/V) ethene.

A	5½ day cultivation, batch
	5-10% (V/V) ethene.
	Total protein 0.27 mg/ml
	Dry weight 1.1 g/l
	Culture 0.53
B	3 day cultivation, batch
	5-10% (V/V) ethene & 0.5% (W/V) glucose.
	Total protein 0.28 mg/ml
	Dry weight 1.17 g/l
	Culture 1.2
C	3 day cultivation, batch
	0.5% (W/V) glucose.
	Total protein 0.23 mg/ml
	Dry weight 1.12 g/l
	Culture 0.7

3.2.8. Stereoselectivity of epoxyalkane degradation

Biological resolution of racemic epoxyalkanes *via* enantioselective destruction of one stereoisomer is an alternative method of obtaining optically pure epoxides. Microsomal epoxide hydrolase has been extensively studied in recent years (Seidegard & DePierre, 1983; Berti, 1986; Wistuba & Schurig, 1986) but, reports on the microbial enzyme(s) are scarce (de Smet *et al.*, 1981a; Weijers *et al.*, 1988b).

Metabolism of racemic 1,2-epoxypropane, 1,2-epoxybutane and *trans*-2,3-epoxybutane (2 mM) by ethene-grown *Micrococcus* sp. M90C (1.3 g/l DW) was followed by head-space analysis on chiral complexation capillary glc. Each isomer was quantified by plotting standard curves of peak areas against sample concentrations (methane as an internal standard). Both enantiomers of 1,2-epoxyalkanes (C₃-C₄) were simultaneously (fig. 25 a & b) hydrolyzed to completion (9-10 hours). A high enantiomeric excess of *R*-1,2-epoxyalkanes (ee=94-96%) therefore did not result from a stereoselective hydrolysis of the *S*-enantiomer, as evident by a constant *R/S* ratio during reaction. This indicates that the microbial production of 1,2-epoxyalkanes is stereospecific.

Complete hydrolysis (24 hours) of the (2*S*,3*S*)-enantiomer of (±) *trans*-2,3-epoxybutane, however, resulted in an enrichment in the (2*R*,3*R*)-enantiomer (fig. 25c). Similarly, it could be argued that the epoxidation reaction is non-stereospecific and a high enantiomeric excess of *trans*-(2*R*,3*R*)-enantiomer (ee=84%) could have resulted from a stereoselective hydrolysis of the (2*S*,3*S*)-enantiomer. If this were the case, a racemic production of epoxide would be expected *initially*, followed by a rapid hydrolysis of one isomer to give the *R*-configuration. The fact that this was not the case, confirms that epoxidation of 1- and 2-olefins occurs stereospecifically.

Chemical synthesis of optically pure *trans*-(2*R*,3*R*)-epoxybutane from (2*S*,3*S*)-tartaric acid involves 8 different steps (Schurig *et al.*, 1980). Kinetic resolution by *Micrococcus* sp. M90C, therefore, provides a simple one step procedure for the preparation of this chiral compound.

Figure 25. Degradation of racemic epoxyalkanes by ethene-grown *Micrococcus* sp. M90C.

Growth condition: 5 $\frac{1}{2}$ day cultivation, batch
5-10% (V/V) ethene.

Reaction: 25 mM phosphate buffer, pH 7.0 at 30°C.
2 mM (final conc.) of racemic epoxides was added to start the reaction.
All incubations were done in triplicate. Analyses were done by complexation glc.

Total protein 0.3 mg/ml
Dry weight 1.3 g/l
Culture OD₆₀₀ 0.54
Resting-cells 6.6

*This may indicate a higher level of enzyme synthesis during the $3\frac{1}{2}$ -4 day period. The bioconversions were carried out under non-optimized conditions (25mM phosphate buffer, pH 7). The specific activities may therefore be an underestimate and would be expected to be significantly higher had they been obtained under optimum pH conditions (5mM glycine buffer, pH 10.5).

3.2.9. Effect of cell age on product formation

Preliminary results indicated that the age of the culture might have some influence on epoxide production by *Micrococcus* sp. M90C. Cells (1-2 g/l DW) were harvested during 3-6 days cultivation on ethene as the sole source of carbon and energy and tested for formation of epoxyethane. Cells were most active when grown for 3½-4 days, whereas six day growth resulted in a four fold reduction in the catalytic activity (table 16).*

Optimization of the culture age has, therefore, improved the initial bioconversion rate. This demonstrates the importance of well-defined growth conditions for epoxide formation.

3.2.10. Substrate specificity

3.2.10.1. Internal & terminal olefins

Ethene-grown *Micrococcus* sp. M90C accumulated (1-13 mM) epoxyalkanes as the sole oxidation product from gaseous olefins (C₂-C₄). *cis*- and *trans*-2-Butenes were epoxidized at significantly lower rates than terminal olefins (fig. 26, table 17). Optimization of the culture age resulted in a 3-5 fold increase in production of 1,2-epoxyalkanes (tables 12 & 17). Given the inaccuracies and the practical difficulties involved in determining microbial consumption of gaseous substrates, bioconversion yields (Y_{P/S}) were based on the amount of olefin fed to the organism. This is, however, an underestimate since the Y_{P/S} values are calculated (at 12th hr. of reaction) when the final epoxide concentration may have not necessarily reached a maximum (fig. 26).

From a stereochemical viewpoint, *cis*-2-butene is expected to be more reactive than the *trans*-isomer. The double bond of *cis*-2-butene is sterically less hindered, and the methyl group is also rotated from planarity and tilted, resulting in a more strained structure than the *trans*-isomer [strain energy: *cis*-2-butene=1.21; *trans*-2-butene=0.05 kcal/mole] (Allinger & Sprague, 1972). Microbial epoxidation of *trans*-2-butene was, however, faster (2 fold) than the *cis*-isomer (table 17). This indicates that the *trans*-configuration is sterically favoured for oxidation in the active site.

Table 16. Effect of culture age on formation of epoxyethane by *Micrococcus* sp. M90C.

Cultivation period (days)	Specific activity*
3	25
3½	50
4	67
4½	45
5	20
5½	16
6	17

* nmole/min./mg protein

Growth condition: Cells were harvested at different stages of growth with 5-10% (V/V) ethene.

Reaction: 25 mM phosphate buffer, pH 7.0 at 30°C.
Bioconversion was started by injecting 10-40% (V/V) ethene.
All incubations were done in triplicate.

Total protein 0.3-0.5 mg/ml
Dry weight 1-2 g/l

1-Octene (C_8) and 1-hexadecene (C_{16}) were also solely epoxidized (but not hydroxylated) to the corresponding 1,2-epoxyalkanes. Doubling carbon chain length from 1-butene to 1-octene/1-hexadecene, reduced the activity markedly (table 17). Each substrate was tested at various concentrations to determine the optimum conditions for its oxidation. Varying the concentration of 1-hexadecene affected the production rate; the optimum value was at 2 mM (fig. 27) and any further increase (up to 5 mM) did not significantly affect production (data not shown). The low YP/s and specific production rate obtained with 1-hexadecene, may be explained by i) lack of substrate consumption and/or ii) steric factors on its interaction in the active site. A relative lack of substrate entry into the cell was evident with its partial utilization, whereas 1-octene was fully consumed within two hours of reaction (fig. 27) despite its low (0.02 mM) solubility (table 1). Steric factors may involve a folding of the saturated end of the olefin (C_8 or C_{16}), resulting from an increased carbon chain length, which might severely affect its entry and subsequent binding to the active site.

The reactivity of a terminal double bond toward epoxidation is affected by the length of the alkyl chain. It is maximal for short chain olefins (C_2 - C_4) but decreases markedly as the chain is lengthened (C_8 - C_{16}). 1-Octene is epoxidized at a similar rate to *trans*-2-butene (table 17). In peracid epoxidation reactions, no significant effect of carbon chain length on epoxidation of a terminal double bond is observed and even propene is as reactive as 1-octene. 2-Butenes are, however, 20 fold more reactive than terminal olefins reflecting the stimulatory effect of alkyl substitution on electrophilic addition to the double bond (Swern, 1947).

3.2.10.2. Cyclic olefins

Epoxyalkanes accumulated (0.2-2 mM) as the sole oxidation products from cyclopentene (C_5) and cyclohexene (C_6). Allylic positions in cyclohexene are secondary and would be more reactive than primary allylic carbons. 2-Cyclohexene-1-ol could, therefore, arise from oxidation of cyclohexene by i) direct hydroxylation of the allylic

carbon or ii) rearrangement of cyclohexene oxide in the active site. To investigate this, ethene-grown cells (2.56 g/1DW) were incubated (30°C) either aerobically with cyclohexene (2 mM), or anaerobically in the presence of cyclohexene oxide (0.2 mM). Neither 2-cyclohexene-1-ol nor cyclohexanone were, however, formed.

Various concentrations of cyclohexene (2-5 mM) did not significantly affect production rate (fig. 28). Cyclohexene was oxidized at a similar rate to *cis*-2-butene. A marked increase (19 fold) in activity was evident with cyclopentene which resulted in a higher final epoxide concentration and YP/s (fig. 28, table 17). The greater solubility of the substrate (C_5 , 8 mM; C_6 , 2.6 mM) may have, however, contributed to the increased rate (table 2).

From a chemical viewpoint, cyclic olefins have a similar, or somewhat higher, reactivity with peracids than open-chain *cis*-alkenes (Swern, 1947). Cyclopentene with its unfavourable bond angle ($\angle=23^\circ$) and angular strain (6.93 kcal/mole) is more reactive than cyclohexene (61° ; 2.61 kcal/mole respectively) [Allinger & Sprague, 1972]. The lower epoxidation rate with cyclohexene may also be explained by steric effects in the product (or in a product-like intermediate). The low steric and angular strain in the cyclohexene ring would be greatly increased on epoxidation which forces the ring to adopt a boat conformation. This is typically 7 kcal/mole higher in energy than the corresponding half-chair conformation in the cyclohexene molecule (Anderson & Roberts, 1970). Apart from the unfavourable torsional strain, cyclohexene oxide may simply be too bulky to be easily accommodated in the active site. It could, therefore, be expected that epoxidation of cyclohexene is much less favourable than open-chain *cis*-alkenes, despite superficial similarity.

The reactivity pattern in the microbial epoxidation of cyclohexene and *cis*-2-butene suggests a similar active site configuration for both substrates. Angular strain combined with the relative lack of steric interactions in cyclopentene oxide, resulted in a rate comparable to propene (table 17).

3.2.10.3. Aryl-substituted olefins

Phenyl glycidyl ether is a common precursor of β -adrenergic receptor blocking agents. These drugs are widely used for the treatment of cardiovascular disorders (Johnstone *et al.*, 1986). Styrene oxide is, however, believed to have mutagenic and carcinogenic properties (Vainio *et al.*, 1976). Only few biocatalysts for production of styrene oxide have been reported to date. These include methanotrophs (Colby *et al.*, 1977; Higgins *et al.*, 1979), a strain of propane-utilizing bacteria (Hou *et al.*, 1983) where only a trace amount was produced, and glucose-grown *Nocardia corallina* (Furuhashi *et al.*, 1986).

Micrococcus sp. M90C accumulated 1,2-epoxyalkanes (~2 mM) in high chemical yields (80-91 mole%), as the sole oxidation product from styrene and phenyl allyl ether (table 17). Control experiments with heat-killed cells indicated that epoxyalkanes (C₂-C₁₆) were produced enzymatically. Reaction mixtures lacking oxygen or substrate were also incapable of product formation. Styrene was epoxidized at a similar rate to propene and cyclopentene, whereas a two fold reduction in activity was observed with phenyl allyl ether (fig. 29, table 17).

Styrene is 17 fold less reactive than cyclopentene in peracid epoxidation reactions. It is, however, 3 fold more reactive than propene due to the increased nucleophilicity of the olefinic system as a result of conjugation of π -electrons of the benzene ring with the double bond (Swern, 1947). Phenyl allyl ether, on the other hand, could be expected to have a similar, if not lower, reactivity to propene due to a mild electron withdrawing effect of oxygen on the σ -bond attached to the methylene group. Therefore, the net effect is a slight reduction in nucleophilicity of the olefinic system.

The substrate specificity of *Micrococcus* sp. M90C toward internal, terminal, cyclic and aryl-substituted olefins is different from that expected on the basis of the chemical reactivity pattern in peracid epoxidation reactions. This indicates some element of enzymatic control which may be exerted by steric constraints on i) substrate interaction in the active site or ii) product formation.

Epoxyalkanes were metabolized, although at very slow rates which allowed the product to accumulate (e.g. 13 mM 1,2-epoxybutane). Non-enzymatic oxidation of epoxides by heat-killed cells was negligible in the standard assay. Further oxidation of the product was evident on prolonged incubations with olefins (figs. 27, 28 & 29). The epoxide-degrading enzyme(s) of *Micrococcus* sp. M90C also appears to have a broad substrate specificity. The slower product formation observed after 1-2 hours of reaction may therefore be explained by the enzymatic oxidation of epoxyalkanes. However, product inhibition can also have an influence (Habets-Crützen & de Bont, 1985).

Table 17. Epoxidation of olefins by ethene-grown *Micrococcus* sp. M90C.

Substrate	Accumulated product	Production rate		Bioconversion yield mole (%)	
		Specific * activity	Specific ** productivity	Fed substrate	Consumed substrate
Ethene	Epoxyethane	67	785	52	ND
Propene	1,2-Epoxypropane	48	563	45	ND
1-Butene	1,2-Epoxybutane	51	601	96	ND
<i>cis</i> -2-Butene	<i>cis</i> -2,3-Epoxybutane	3	36	8	ND
<i>trans</i> -2-Butene	<i>trans</i> -2,3-Epoxybutane	7	86	11	ND
1-Octene	1,2-Epoxyoctane	8	94	23	23
1-Hexadecene	1,2-Epoxyhexadecane	0.05	0.6	0.3	2
Cyclopentene	Cyclopentene oxide	37	438	41	60
Cyclohexene	Cyclohexene oxide	2	25	7	8
Styrene	Styrene oxide	38	445	90	91
Phenyl allyl ether	Phenyl glycidyl ether	18	214	80	89

* nmole/min./mg protein
** μ mole/hr./g. DW

Bioconversion yield ($Y_{P/S}$) is based on the amount of substrate *fed* to or *consumed* by the organism.

ND not determined

Growth condition: 4 day cultivation, batch
5-10% (V/V) ethene.

Reaction: 25 mM potassium phosphate buffer, pH 7.0 at 30°C.
Reaction was started by adding gaseous/liquid olefins at various optimum concentrations (see text).

Total protein 0.5 mg/ml
Dry weight (DW) 2.56 g/l
OD₆₀₀ Culture 0.48
Resting-cells 15.0

Figure 26. Production of epoxides from gaseous olefins by *Micrococcus* sp. M90C.

- A 1,2-epoxybutane
- B epoxyethane
- C 1,2-epoxypropane
- D *trans*-2,3-epoxybutane
- E *cis*-2,3-epoxybutane

Aqueous samples were directly analyzed by glc in triplicate.
Reaction was started by injecting 10-40% (V/V) of gaseous olefins.

Figure 27. Epoxidation of 1-hexadecene and 1-octene by *Micrococcous* sp. M90C.

● Product formation
○ Substrate disappearance

Samples were extracted into ether and analyzed by glc.
All incubations were done in triplicate.
Bioconversion was started by adding 1-hexadecene to a final concentration of A) 2 mM or B) 1 mM and 1-octene at 2 mM.

Figure 28. Epoxidation of cyclopentene and cyclohexene by *Micrococcus* sp. M90C.

● Product formation
○ Substrate disappearance

Samples were extracted into ether and analyzed by glc.
All incubations were done in triplicate.
Bioconversion was started by adding 5 mM cyclopentene.
Cyclohexene was added to a final concentration of A) 5 mM or B) 2 mM.

Figure 29. Epoxidation of phenyl allyl ether and styrene by *Micrococcus* sp. M90C.

- Product formation
- Substrate disappearance

Samples were extracted into ether and analyzed by glc.
 All incubations were done in triplicate.
 Bioconversion was started by adding phenyl allyl ether and styrene to a final concentration of 2 mM.

3.2.11. The involvement of a specific monooxygenase

Cell-free extracts (0.8-1.26 mg/ml protein) of ethene-grown *Micrococcus* sp. M90C were used to investigate the involvement of a monooxygenase in the production of epoxyalkanes. Formation of epoxyethane by the soluble fraction was dependent on the presence of molecular oxygen and NADH. This is indicative of a typical monooxygenase reaction (Colby *et al.*, 1977; de Bont *et al.*, 1979). No activity was observed in the absence of molecular oxygen, NADH or with heat-killed extracts. The majority of activity (90%) was localized in the soluble fraction. Particulate fractions also catalyzed the formation of epoxyethane in a NADH-independent reaction that required molecular oxygen for activity (table 18). Exogenous addition of NADH (5 mM), however, completely inhibited the particulate activity. Both fractions (soluble & particulate) accumulated (0.5-1.2 mM) epoxyethane as the sole oxidation product and were unable to hydroxylate alkanes or alkenes. This is in accordance with similar findings in whole cells.

The ethene monooxygenase (EMO) is believed to be present in two forms. The soluble EMO requires molecular oxygen and is NADH-dependent. The other form of the enzyme appears to be membrane-bound and does not require NADH as an electron donor.

The effect of stabilizing agents such as β -mercaptoethanol (BME) on the monooxygenase was studied. BME (5 mM) completely inhibited the soluble activity in the presence of NADH, whereas the particulate activity was only halved (fig. 30, table 18). The monooxygenase activity was stable to short-term (7-10 days) storage (-40°C) in the absence of any stabilizing agents.

The soluble EMO also catalyzed the stereospecific formation of *R*-1,2-epoxypropane (ee=95%), *R*-1,2-epoxybutane (ee=92%) and *trans*-(2*R*,3*R*)-epoxybutane (ee=83%) in the presence of NADH and molecular oxygen (table 19). This is consistent with the involvement of an EMO in the production of chiral epoxides in whole cells.

Figure 30. Epoxidation of ethene by cell-free extracts of *Micrococcus* sp. M90C.

Table 18. Production of epoxyethane by whole cells and cell-free extracts of *Micrococcus* sp. M90C.

Fractions	Specific * activity	Relative activity (%)
Whole cells	60	100
S 48 with NADH without BME	19	32
S 48 without NADH and BME	0	0
P 48 without NADH and BME	2	3
P 48 without NADH with BME	1	2

* nmole/min./mg protein

No epoxidation activity was found in the soluble fraction without NADH. BME completely inhibited soluble activity in the presence of NADH. Activity in particulate fractions with or without BME was NADH-independent.

Table 19. Enantiomeric composition of epoxyalkanes by whole cells and cell-free extracts of *Micrococcus* sp. M90C.

Accumulated product	Enantiomeric excess (%)	
	Whole cells	Cell-free extracts
<i>R</i> ,1-2-Epoxypropane	96	95
<i>R</i> ,1-2-Epoxybutane	94	92
<i>trans</i> -(2 <i>R</i> ,3 <i>R</i>)-Epoxybutane	84	83

Enantiomeric excess (ee) = $\frac{R-S}{R+S} \times 100$

Values in relative %, (±1%) as analyzed by chiral complexation capillary glc.
All incubations were done in triplicate.

Reaction: 25mM phosphate buffer, pH 7.0 at 30°C.
Bioconversion was started by injecting 10-40% (V/V) olefins.

3.2.12. Asymmetric sulphur oxygenation

3.2.12.1. Introduction

Optically active sulfoxides constitute a group of useful chiral auxiliary compounds for asymmetric synthesis (Solladié, 1981; Posner *et al.*, 1982). The most practical synthetic method to obtain high optical purity sulfoxides involves the reaction of a resolved sulphinate ester with a Grignard reagent. The synthetic utility of this method is, however, limited because in many cases chiral sulfoxides in a moderate or low yield are obtained, depending on the structure and availability of both starting materials. Additionally, reaction conditions must be carefully selected, otherwise considerable quantities of sulphides and other impurities (that are often difficult to separate from the desired sulfoxide) are produced (Drabowicz *et al.*, 1982). Asymmetric oxidation of sulphides with optically active peroxides in the presence of bovine serum albumin (as a chiral binding site), also results in a rather low enantiomeric excess (Sugimoto *et al.*, 1979 a & b; Ogura *et al.*, 1980).

Biological oxygenation of thioethers is an alternative and effective method to obtain chiral sulfoxides because of i) stereospecificity and ii) selectivity, i.e., it allows formation of sulfoxides in the presence of other functionalities which would be labile to chemical oxidation. Actively grown or 'resting-cells' of fungi have been widely used to obtain sulfoxides in good optical yields (Auret *et al.*, 1968; Abushanab *et al.*, 1978; Holland *et al.*, 1985). Other methods of biotransformation such as the use of enzymes of mammalian origin (Waxman *et al.*, 1982; Takata *et al.*, 1983) or peroxide oxidation in the presence of enzyme templates (Sugimoto *et al.*, 1981) are, however, not generally applicable to preparative scale work.

Bacterial production of chiral sulfoxides include, biotransformation with: i) *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* (Entsch *et al.*, 1976), ii) purified cyclohexanone monooxygenase from *Acinetobacter* (Light *et al.*, 1982), iii) growing cells of *Corynebacterium equi* IFO 3730 (Ohta *et al.*, 1984) and iv) a partially purified non-haem iron monooxygenase from *Pseudomonas oleovorans* (May & Katopodis, 1986). Cyclohexanone monooxygenase forms *S*-(-)-sulfoxide (ee=64%) from 4-tolyl

ethyl sulphide. In contrast, incubation of aryl alkyl sulphides and aryl allyl sulphides with growing cells of *C. equi* results in optically active *R*-(+)-sulphoxides (ee=75-100%). Chemical yields of sulphoxide vary (25-100 mole%) considerably, with overoxidation to sulphone being a problem in some cases. There is, however, no report to date on heteroatom oxygenations by gaseous alkene-utilizing bacteria.

3.2.12.2. Production of phenyl methyl sulphoxide

Ethene-grown *Micrococcus* sp. M90C (2.56 g/l DW) catalyzed oxygenation of thioanisole (3.25 mM). Control experiments i) with heat-killed cell suspensions and ii) lacking oxygen or substrate were incapable of sulphoxide formation. Product identification was done by retention time comparisons, co-chromatography with authentic standards, ¹H NMR and mass spectrometry. Phenyl methyl sulphoxide accumulated (3.2 mM) as the sole oxidation product (fig. 31). The specific production rate (non-optimized) and Y_{P/S} (99 mole %) were very impressive (table 20). Phenyl methyl sulphoxide was metabolized to the corresponding sulphone, although at a very slow rate which allowed the product to accumulate. Non-enzymatic oxidation of sulphoxide by heat-killed cells was negligible in the standard assay. Further oxidation of the product was also evident on prolonged incubations with thioanisole (fig. 31). At present, it remains uncertain whether the same enzyme catalyzes the oxidation of phenyl methyl sulphoxide and epoxyalkanes. However, the involvement of more than one enzyme system cannot be excluded.

The mass spectrum of the microbial product was identical with the structure of the chemically synthesized (±) phenyl methyl sulphoxide (fig. 32). Electron impact: molecular ion (M) at m/e=140 characteristic of the expected mass, base peak at 51 (C₄H₃⁺) and peaks at 125 (M-15, methyl loss), 109 (C₆H₅S⁺), 97 (M-43, ring cleavage & methyl loss), 91 (C₇H₇⁺, tropylium ion), 77 (C₆H₅⁺, phenyl cation), 65 (C₅H₅⁺, acetylene loss from C₇H₇⁺) and 39 (C₃H₂⁺, ring cleavage) compared well in intensities with the authentic standard.

● [Phenyl methyl sulfoxide]
○ [Thioanisole]

Figure 31. Formation of phenyl methyl sulfoxide by *Micrococcus* sp. M90C.

Growth condition: 4 day cultivation, batch
5-10% (V/V) ethene.

Reaction: 25 mM phosphate buffer, pH 7.0 at 30°C.
Bioconversion was started by adding 3.25 mM thioanisole.
Samples were extracted into ether and analyzed by glc.
All incubations were done in triplicate.

Total protein 0.5 mg/ml
Dry weight 2.56 g/l

OD₆₀₀ Culture 0.48
Resting-cells 15.0

Table 20. Catalytic activity of *Micrococcus* sp. M90C for the production of phenyl methyl sulphoxide.

Specific activity *	Specific productivity **	Bioconversion yield (mole %)	
		Fed	Consumed
53	617	98.5	99.5

Bioconversion yield (Y_{PS}) is based on the amount of substrate *fed* to or *consumed* by the organism.

* nmole/min./mg protein

** μ mole/hr./g. DW

Figure 32. Electron impact mass spectra of phenyl methyl sulphoxide.

A) Racemic

B) *Micrococcus* sp. M90C

3.2.12.3. Optical purity of microbial sulphoxidation

Microbial production of phenyl methyl sulfoxide was scaled-up (18 fold) to yield up to 20 mg for chiral analysis. After two hours of incubation with thioanisole, ethene-grown cells were removed by centrifugation, the supernatant extracted with equal volumes of ether and the final residue dissolved in benzene- d_6 . This was essentially devoid of any impurity, with no overoxidation to sulphone detected, as analyzed by ^1H NMR and mass spectrometry.

The enantiomeric composition of the product was determined by ^1H NMR in the presence of the europium shift reagent $[\text{Eu}(\text{hfc})_3]$ in a 1:5 ratio (Egan *et al.*, 1981). Without $\text{Eu}(\text{hfc})_3$, the NMR spectra of microbial and ($\pm$) phenyl methyl sulfoxide were identical (fig. 33a) [chemical shifts (ppm) : $\delta=2.07$ (singlet, 3 methyl protons), $\delta=7.03$ (multiplet, 3 *meta* & *para* protons) and $\delta=7.4$ (double of doublet, 2 *ortho* protons)]. The line at 7.15 ppm is due to benzene- d_6 (chemical shift reference). On addition of $\text{Eu}(\text{hfc})_3$, (0.2 molar equivalent of CSR), the methyl singlet in the ($\pm$) phenyl methyl sulfoxide shifted downfield and was split into two distinct peaks of equal intensity (fig. 33b). To achieve this, the concentration of $\text{Eu}(\text{hfc})_3$ was varied to obtain a near base-line separation (*inset*, fig. 33b). Additionally, the aromatic hydrogen signals (C_2, C_6 *ortho* position) separated further and moved downfield ($\delta=10$). The microbial product was, however, shown to be enantiomerically pure ($ee>98\%$), as only one envelope of signal was detected from the methyl protons (fig. 33c).

The optically pure product gave a positive rotation $[\alpha]_{\text{D}}^{20}=+119.7^\circ$ ($c=1.35$, ethanol), whereas no rotation was observed with the racemic sulfoxide solution. This is compared to the literature value of $[\alpha]_{\text{D}}^{25}=+128.5^\circ$ ($c=1.5$, ethanol) for the asymmetric synthesis of optically pure *R*-(+)-phenyl methyl sulfoxide (Wudl & Lee, 1972). The absolute configuration of the microbial product is considered to be *R*, as evident by a positive sign of optical rotation.

The further metabolism of the product may or may not be enantioselective. It could

therefore be argued that production is non-stereospecific and a high enantiomeric excess of *R*-(+)-phenyl methyl sulphoxide could have resulted from an enantioselective hydrolysis of the *S*-enantiomer. If this were the case, a racemic product would be expected *initially* followed by a rapid hydrolysis of one isomer to give an enrichment in the *R*-configuration. Only one enantiomer was, however, initially observed and the optical purity remained unchanged throughout the reaction, as analyzed by chiral-shift NMR. Similarly, the possibility of an isomerization process can be discounted. The microbial formation of *R*-(+)-phenyl methyl sulphoxide is, therefore, stereospecific and its enantiomeric excess is higher than any previous report (table 21).

Production of phenyl methyl sulphoxide in high chemical and optical yields, provides an attractive and convenient method for its preparation in milligram quantities. The stereospecificity of oxygen transfer, suggests that the EMO of *Micrococcus* sp. M90C may be involved in chiral production of phenyl methyl sulphoxide as well as epoxyalkanes. This demonstrates its ability as a 'general purpose' biocatalyst for the production of optically pure oxygenated compounds.

Bruker 270 MHz

Me methyl group

CSR $Eu(hfc)_3$

C_6D_6 benzene- d_6

Figure 33. Proton NMR spectra of phenyl methyl sulfoxide.

A) Racemic

B) Racemic plus 0.2 molar equivalent of CSR

C) Produced by *Micrococcus* sp. M90C plus 0.4 molar equivalent of CSR

Table 21. Reported optical purities of microbial phenyl methyl sulphoxide.

Organism	Enantiomeric excess (%)	Absolute configuration	Reference
<i>Corynebacterium equi</i>	75	<i>R</i>	Ohta <i>et al.</i> (1984)
<i>Mortierella isabellina</i>	58	<i>R</i>	Holland <i>et al.</i> (1985)
<i>Micrococcus</i> sp. M90C	>98	<i>R</i>	This work.

3.3. Biocatalysts for production of chiral epoxides

Initial studies resulted in the selection of 18 bacteria that grew well in liquid medium with ethene or propene as the sole source of carbon and energy. The organisms included *Aerococcus*, *Alcaligenes*, *Micrococcus* and *Staphylococcus* spp. and a number of Gram-negative, Gram-positive and Gram-variable mesophilic rods/coccobacilli not yet identified. *Micrococcus* sp. M90C catalyzed the stereospecific heteroatom oxygenation as well as epoxidation of a wide range of olefins in high chemical and optical yields. Preliminary data showed that the remaining 17 organisms also accumulated epoxyalkanes from 1-alkenes (C_2 - C_4) and were, therefore, screened for i) pattern of hydrocarbon utilization and ii) production of optically active epoxides. Controls with heat-killed cells or lacking oxygen/substrate were incapable of product formation.

3.3.1. Growth characteristics on hydrocarbons

Ethene- and propene-utilizing bacteria were tested for growth on a range of gaseous saturated and unsaturated hydrocarbons (table 22). The concentration of the hydrocarbons was 5-10% (V/V), whilst that of the epoxides was 0.2% (V/V) and other carbon sources was 0.5% (W/V).

The pattern of substrate utilization indicated the presence of two groups of organisms: bacteria that grew i) only on olefins or ii) on both alkanes and alkenes. All isolates grew well (3-6 days) on their isolation substrates and on carbohydrates (glucose, pyruvate, succinate) but not on methane. Growth on epoxides, *cis*- and *trans*-2-butenes and 1,3-butadiene was evident after a long lag. Ethene did not support growth of propene-utilizers but 1-butene did. Growth of ethene-utilizers on propene and/or 1-butene varied, and was dependent on the organism used. In general, growth on epoxides was correlated with growth on the corresponding olefins.

Table 22. Pattern of hydrocarbon utilization by ethene- and propene-grown bacteria.

Isolate No. M	25	26	49	67	90C	93A	94A	97B	99	101	185	186	122	137	142	156	162	189K
Growth substrate	Ethene-utilizers:												Propene-utilizers:					
Ethene	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
Propene	+	+	-	-	+	+	-	+	+	-	-	-	+	+	+	+	+	+
1-Butene	-	+	+	+	+	-	-	-	-	-	+	-	+	+	+	+	+	+
<i>trans</i> -2-Butene	-	+	-	-	-	+	-	+	-	-	-	-	+	+	+	+	-	-
<i>cis</i> -2-Butene	-	+	-	-	-	+	-	+	-	-	-	-	+	+	+	-	-	-
Butadiene	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Methane	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethane	+	-	-	-	-	-	+	-	+	+	-	+	-	-	-	-	-	-
Propane	+	-	-	-	-	-	+	-	+	+	-	+	-	-	-	+	-	-
Butane	+	-	-	-	-	-	+	-	+	+	-	+	-	-	-	+	-	-
Epoxyethane	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
1,2-Epoxypropane	+	+	-	-	+	+	-	+	+	-	-	-	+	+	+	+	+	+
1,2-Epoxybutane	-	+	-	+	+	-	-	-	-	-	+	-	+	+	+	+	+	+

Gaseous hydrocarbons: 5-10% (V/V). Epoxides: 0.2% (V/V).

All flasks were in duplicate. Erlenmeyer flasks with no substrate served as controls.

Growth (+) or no growth (-) was determined during four weeks of incubation at 30 °C.

3.3.2. Enantiomeric excess of lower epoxyalkanes

Ethene- or propene-grown cells (1-4 g/l DW) stereoselectively formed *R*-1,2-epoxypropane (ee=90-96%), *R*-1,2-epoxybutane (ee=90-98%) and *trans*-(2*R*,3*R*)-epoxybutane (ee=64-88%) [table 23]. Both enantiomers of a racemic solution (2mM) of 1,2-epoxyalkane (C₃-C₄) were *slowly* hydrolyzed (8-15 hr. for complete hydrolysis) and the *R/S* ratio remained constant during the reaction (data not shown). This indicates that the microbial epoxidation of terminal olefins occurs stereospecifically. The (2*S*,3*S*)-enantiomer of (±)*trans*-2,3-epoxybutane (2mM) was, however, enantioselectively hydrolyzed (20-36 hr. for complete hydrolysis) to give the (2*R*,3*R*)-enantiomer (data not shown). If epoxidation of *trans*-2-butene were non-stereospecific, a racemic production of epoxide would be expected *initially*, followed by a *rapid* hydrolysis of one isomer to give an enrichment in the *R*-configuration. The fact that this was not the case, confirms that 1- and 2-olefins are epoxidized stereospecifically.

As a possible explanation for the lower enantiomeric excess obtained with *trans*-2-butene, it is proposed that the steric hinderance of the bulky methyl groups in the substrate may alter its orientation in the active site in such a way as to decrease the stereospecificity of microbial epoxidations. Enantiomeric compositions of *trans*-2,3-epoxybutane formed by propene-utilizers (ee=64-70%), were lower than ethene-utilizers (ee=76-88%). It can be argued that the *trans*-2-butene molecule, is allowed a greater degree of movement within the active site of the enzyme found in the propene-utilizers. These organisms (propene-utilizers) might ultimately prove to be unsuitable for production of optically pure 2,3-epoxyalkanes. The propene-grown isolate M142, however, enantioselectively hydrolyzed racemic *trans*-2,3-epoxybutane in a similar manner to *Micrococcus* sp. M90C, thus enabling the synthesis of optically pure *trans*-(2*R*,3*R*)-epoxybutane (data not shown).

Aerococcus sp. M186, capable of growth on both gaseous alkanes and alkenes, excreted optically active *R*-1,2-epoxypropane (ee=94%) and *R*-1,2-epoxybutane (ee=94%) when grown on ethene. Ethane-grown cells, however, excreted racemic 1,2-epoxyalkanes. The difference in the enantiomeric excess suggests that the organism may contain two

enzyme systems. One may be an 'alkene monooxygenase' induced by growth of the organism on ethene, can only epoxidize olefins and is incapable of hydroxylation of alkanes/alkenes. The other enzyme is probably an 'alkane-type monooxygenase' that epoxidizes olefins and hydroxylates alkanes as well as alkenes, but is only induced by growth of the organism on ethane. This is similar to findings of Habets-Crützen *et al.* (1985) in ethane- or ethene-grown *Mycobacterium* E20. The optical purities of *R*-1,2-epoxypropane (ee=76%) and *R*-1,2-epoxybutane (ee=62%) in alkene-grown cells are, however, much lower than those in *Aerococcus* sp. M186, suggesting that there may be differences in the active site of the enzymes involved.

Table 23. Enantiomeric composition of microbial epoxidations*.

Bacterial strain	1,2-Epoxypropane			1,2-Epoxybutane			<i>trans</i> -2,3-Epoxybutane		
	<i>R</i>	<i>S</i>	ee	<i>R</i>	<i>S</i>	ee	2 <i>R</i> ,3 <i>R</i>	2 <i>S</i> ,3 <i>S</i>	ee
M 25	97	3	94	98	2	96	90	10	80
M 26	96	4	92	96	4	92	91	9	82
M 49	97	3	94	98	2	96	92	8	84
M 67	97	3	94	98	2	96	90	10	80
M 90C	98	2	96	97	3	94	92	8	84
M 93A	98	2	96	99	1	98	93	7	86
M 94A	96	4	92	96	4	92	90	10	80
M 97B	98	2	96	97	3	94	90	10	80
M 99	97	3	94	95	5	90	89	11	78
M 101	96	4	92	95	5	90	88	12	76
M 185	97	3	94	98	2	96	91	9	82
M 186	97	3	94	97	3	94	94	6	88
M 122	96	4	92	98	2	96	85	15	70
M 137	96	4	92	98	2	96	84	16	68
M 142	96	4	92	96	4	92	82	18	64
M 156	95	5	90	98	2	96	84	16	68
M 162	95	5	90	98	2	96	84	16	68
M 189K	96	4	92	96	4	92	85	15	70

* Values in relative %, ±1%.

All strains were alkene-grown, enantiomeric excess(ee) was determined by chiral complexation glc.

All incubations were done in triplicate.

$$ee\% = \frac{R-S}{R+S} (100)$$

Reaction: 25mM potassium phosphate buffer, pH = 7.0 at 30°C.

All incubations were done in triplicate.

[Gaseous olefins] = 10-40% (V/V)

3.4.

Beta-blockers

3.4.1. Bacterial epoxidation of phenyl allyl ether

β -Adrenergic receptor blocking agents (beta-blockers) are a group of compounds widely used for the treatment of cardiovascular disorders. The majority have a common structural feature of a 3-aryloxy- or a 3-heteroaryloxy-1-alkylamino-2-propanol. Although these drugs have historically been made as racemates, there is an increasing tendency to market the single enantiomers (e.g. Metoprolol, Atenolol, Propranolol) because the biological activity mainly resides in the *S*-stereoisomer (Johnstone *et al.*, 1986; Phillips *et al.*, 1988). Preparation of optically active beta-blockers generally involves chemical resolutions or rather lengthy chemical syntheses from chiral precursors (Weinstock *et al.*, 1976). An alternative route for obtaining optically pure beta-blockers is *via* stereoselective microbial epoxidation of aryl allyl ethers to give *S*-(+)-aryl glycidyl ethers, which are reacted with amines to give *S*-(-)-3-substituted-1-alkylamino-2-propanols [active components of beta-blockers] (fig. 34). In view of the biological importance of these compounds, the catalytic activity of ethene- and propene-utilizing bacteria for epoxidation of phenyl allyl ether was investigated.

Alkene-grown cells (1-4 g/l DW) accumulated (0.4-1.7 mM) phenyl glycidyl ether as the sole product of olefin oxidation (figs. 35 & 36) with specific production rates (non-optimized) in the range 69-323 μ mole/hr./g.DW (table 24). Control experiments i) with heat-killed cells and ii) lacking oxygen or substrate were incapable of epoxide formation. Structure identification and purity of the product was done by retention time comparisons, co-chromatography with authentic standards, acid hydrolysis (May & Abbott, 1973), chiral-shift ^1H NMR, HPLC and mass spectrometry. The product was slowly metabolized by ethene-utilizers, whereas all propene-utilizers (except M156) exhibited significant further oxidation which would contribute to their lower conversion yield. Non-enzymatic hydrolysis of epoxide by heat-killed cells was negligible in the standard assay.

Figure 34. Synthetic route to *S*-(-)-3-aryloxy-1-alkylamino-2-propanols incorporating stereospecific microbial epoxidation (Johnstone *et al.*, 1986).

Table 24. Production of phenyl glycidyl ether by various alkene-utilizing bacteria.

Bacterial strains	Growth* substrate	Growth (days)	OD ₆₀₀	Total protein	Dry weight	Specific productivity μmole/hr /g.DW	Specific activity nmole/min./mg protein	Product yield ** mole (%)	
				mg/ml	g/l			Fed alkene	Consumed substrate
M26	Ethene	4	0.50	0.40	1.57	321	21	80	89
M49	Ethene	6	0.20	0.70	4.17	71	7	20	40
M90C	Ethene	4	0.48	0.50	2.56	214	18	80	89
M93A	Ethene	4	0.61	0.35	1.56	323	24	85	85
M94A	Ethene	5	0.10	0.20	1.81	73	11	20	20
M97B	Ethene	4	0.85	0.62	2.22	318	19	70	74
M185	Ethene	6	0.15	0.52	4.08	69	9	28	50
M186	Ethene	5	0.48	0.65	1.83	192	9	45	56
M122	Propene	4	0.44	0.75	2.43	204	11	33	54
M137	Propene	5	0.46	0.83	2.88	191	11	28	55
M142	Propene	5	0.41	0.74	1.75	274	11	38	60
M156	Propene	5	0.40	0.66	2.15	184	10	23	44
M162	Propene	5	0.69	0.80	1.36	282	8	35	58
M189K	Propene	4	0.48	0.70	1.71	226	9	25	54
M25	Ethene	5	0.20	Not active					
M67	Ethene	6	0.21						
M99	Ethene	6	0.11						
M101	Ethene	5	0.15						

Samples were analyzed in triplicate by glc.

* Alkenes were supplied at a concentration of 5-10% (V/V).

** Bioconversion yield (Y P/S) is based on the amount of substrate *fed* to or *consumed* by each organism.

Reaction: 25mM potassium phosphate buffer, pH = 7.0 at 30°C.
All incubations were done in triplicate.
[Phenyl allyl ether] = 2mM

Samples were extracted into ether and analyzed by glc in triplicate.
 Bioconversion was started by adding 2mM (final conc.) phenyl allyl ether.

Figure 35. Production of phenyl glycidyl ether by ethene-utilizing bacteria.

Samples were extracted into ether and analyzed by glc in triplicate.
Bioconversion was started by adding 2mM (final conc.) phenyl allyl ether.

Figure 36. Production of phenyl glycidyl ether by propene-utilizing bacteria.

The mass spectra of the three microbial (M26, M90C, M93A) products were identical with the structure of the chemically synthesized ($\pm$) phenyl glycidyl ether (fig. 37). Electron impact: molecular ion (M) at $m/e=150$ characteristic of the expected mass, base peak at 29 (CHO^+) and peaks at 146 ($\text{C}_6\text{H}_5\text{OC}_3\text{HO}^+$, hydrogen loss), 120 ($\text{C}_6\text{H}_5\text{OC}_2\text{H}_3^+$, formaldehyde loss), 107 ($\text{M}-43$, ring cleavage & $\text{C}_2\text{H}_3\text{O}$ loss), 94 ($\text{C}_6\text{H}_5\text{O}^+\text{H}$), 91 (C_7H_7^+ , tropylium ion), 77 (C_6H_5^+ , phenyl cation), 66 (C_3H_6^+), 65 (C_5H_5^+ , acetylene loss from C_7H_7^+), 57 (C_3OH_5^+ , loss of $\text{C}_6\text{H}_5\text{O}$), 51 (C_4H_3^+ , acetylene loss from C_6H_5^+), 39 (C_3H_3^+ , ring cleavage) and 31 ($\text{CH}_2\text{O}^+\text{H}$) compared well in intensities with the authentic standard.

3.4.2. Enantiomeric excess of phenyl glycidyl ether

In addition to *Micrococcus* sp. M90C, two other ethene-utilizing bacteria (M26, M93A) which had the highest conversion yields ($\text{Y}_{\text{P/S}}=85\text{-}89$ mole%), the highest rates of product formation and the highest final epoxide concentrations, were selected for scale-up (28 fold). This yielded up to 54 mg of epoxide for analysis by chiral-shift NMR and HPLC. After three hours of incubation with phenyl allyl ether, ethene-grown cells were removed by centrifugation, the supernatant extracted with equal volumes of ether and the final residue dissolved in acetone- d_6 . This was essentially devoid of any impurity, with no epoxide hydrolysis detected, as analyzed by ^1H NMR and mass spectrometry.

The enantiomeric composition of microbial epoxides was determined by their complexation with the chiral-shift reagent, $\text{Eu}(\text{hfc})_3$ and analysis of ^1H NMR spectra. Without $\text{Eu}(\text{hfc})_3$, the NMR of microbial and ($\pm$) phenyl glycidyl ether were identical (fig. 38) [chemical shifts (ppm): $\delta=2.1$ (acetone- d_6), $\delta=2.7$ (double of doublet, H_c), $\delta=2.85$ (double of doublet, H_d), $\delta=3.3$ (multiplet, H_e), $\delta=3.9$ (double of doublet, H_b), $\delta=4.3$ (double of doublet, H_a), $\delta=6.95$ (multiplet, 3 *meta* & *para* protons) and $\delta=7.3$ (double of doublet, 2 *ortho* protons)]. On addition of $\text{Eu}(\text{hfc})_3$, (0.125 molar equivalent of CSR), the envelope of signals from each of the geminal protons in the racemic ($\pm$) phenyl glycidyl ether shifted downfield (H_a , 4.8; H_b , 4.35 ppm) and were each split into two envelopes of signals of equal intensity (spectra not shown), whereas the envelope of signals from the geminal

protons in the microbial product was split in the ratio 19:1 (an optical purity of at least 90%).

Further confirmation of the optical purity of products was done by HPLC. The *R*- and *S*-enantiomers of phenyl glycidyl ether were directly separated by injecting ether extracts of the microbial and racemic epoxide on to a CHIRALCEL-OD column. Each isomer was identified by co-injection and retention time comparisons with pure authentic specimens and quantified by plotting standard curves of peak areas against sample concentrations. The *R*- and *S*-enantiomers were present in equal amounts in the racemic chemically synthesized epoxide, whereas each of the three ethene-utilizers produced phenyl glycidyl ether almost exclusively (ee=93%) in the *S*-configuration (fig. 39).

The absolute configuration of the microbial product was confirmed by optical rotation measurements as evident by a positive sign of rotation (table 25), whereas no rotation was observed with the ($\pm$) phenyl glycidyl ether. By comparison, Johnstone *et al.* (1986) reported $[\alpha]_D^{25} = +11.38^\circ$ ($c=1.09$, ethanol) for the enzymatic synthesis of *S*-(+)-phenyl glycidyl ether.

Microbial epoxidation of phenyl allyl ether occurred stereospecifically because *initially* the *S*-enantiomer was predominantly formed and the chirality remained unchanged during the reaction, as analyzed by chiral-shift NMR. Similarly, the possibility of an isomerization process can be discounted. The enantiomeric excess of *S*-(+)-phenyl glycidyl ether, produced by each of the three ethene-utilizers, is higher than any previous report (table 26). This has attracted considerable industrial interest and is currently under investigation at ICI for commercial production of optically active beta-blockers.

Figure 37. Electron impact mass spectra of phenyl glycidyl ether.

A) Racemic

B) *Micrococcus* sp. M90C

Figure 38. Proton NMR spectrum of microbial phenyl glycidyl ether.

HPLC traces of the three ethene-utilizers (M26, M90C, M93A) were similar.

Figure 39. Enantiomeric resolution of phenyl glycidyl ether by HPLC.

A) Racemic

B) *Micrococcus* sp. M90C

Table 25. Optical rotations and enantiomeric compositions of microbial phenyl glycidyl ether.

Bacterial strains	Growth substrate	$[\alpha]_D^{20}$	$[c]^*$	**ee	Absolute configuration
M 26	Ethene	+10.63°	1.40	93.1	S
M 90C	Ethene	+11.1°	1.20	93.4	S
M 93A	Ethene	+10.85°	1.31	93.3	S

* Concentration (g/100 ml) in ethanol.

** Enantiomeric excess (ee) was determined by HPLC on CHIRALCEL-OD column and expressed as a percentage:

$$ee\% = \frac{S-R}{S+R} (100)$$

Table 26. Reported chiralities of phenyl glycidyl ether by various bacteria.

Organism	Enantiomeric excess (%)	Absolute configuration	Reference
<i>Nocardia corallina</i> (ATCC 31338)	67	S	Furuhashi & Takagi (1985)
<i>Arthrobacter rubellus</i> (ATCC 21495)	49	ND	Furuhashi & Takagi (1985)
<i>Mycobacterium</i> WMI (NCIB 11626)	74	S	Johnstone <i>et al.</i> (1986)
<i>Micrococcus</i> sp. M90C	93	S	This work.

ND not determined

3.5. A comparison of substrate specificities of alkene-utilizing bacteria

All of the 18 ethene- and propene-utilizing bacteria formed *R*-1,2-epoxyalkanes with a very high degree of stereoselectivity (ee=90-98%). The majority of these organisms (14 strains) also accumulated phenyl glycidyl ether. The three ethene-utilizers (M26, M90C, M93A) each catalyzed the stereospecific formation of *S*-(+)-phenyl glycidyl ether (ee=93%) as a precursor for chiral production of beta-blockers. In addition to *Micrococcus* sp. M90C, the substrate specificities of 4 ethene- and 2 propene-utilizers, representing a range of genera, were investigated in order to probe the active site of the enzymes involved. The substrates used were internal (*trans*-2-butene), terminal (1-butene, 1-octene) and cyclic (cyclohexene) olefins as well as styrene. Alkene-grown cells (1-3 g/l DW) accumulated epoxyalkanes as the sole product (no hydroxylation) of olefin oxidation. Controls with heat-killed cell suspensions or lacking oxygen/substrate were incapable of product formation. Identification was done by retention time comparisons, co-chromatography with authentic standards and acid hydrolysis (May & Abbott, 1973).

3.5.1. Internal & terminal olefins

As a group, the four ethene- (M26, M90C, M93A, M186) and two propene- (M142, M156) utilizers showed similar reactivity pattern toward epoxidation of the terminal double bond. Activity was maximal for 1-butene but decreased markedly (3-6 fold) when the chain doubled, whereas production rate was unaffected by carbon chain length in *Staphylococcus* sp. M97B, and even 1-octene was as reactive as 1-butene (table 27). Highest specific production rate, highest bioconversion yield ($Y_{P/S} = 96$ mole %) and highest final 1,2-epoxybutane concentration was obtained with *Micrococcus* sp. M90C. (figs. 40 & 41, table 27). The internal double bond of *trans*-2-butene was also epoxidized although at rates lower than 1-butene, whereas a 2 fold increase in activity was evident with *Staphylococcus* sp. M97B (fig. 42, table 27) resulting in a higher final epoxide concentration (7.5 mM).

*Whole cell biotransformations may be affected by i) substrate entry into and product permeation out of the cell and ii) undesirable side reactions which may take place due to the presence of other enzyme systems. It should be noted that data obtained with whole cell studies may not truly reflect reactions at the enzymatic level and any such extrapolations may therefore be open to speculation.

3.5.2. Cyclohexene

Analogous to the situation in *Micrococcus* sp. M90C, none of the organisms formed 2-cyclohexene-1-ol aerobically from cyclohexene (by direct hydroxylation of the secondary allylic carbon) or anaerobically from cyclohexene oxide (by rearrangement in the active site). Cyclohexanone was also not detected during the reaction. Cyclohexene was fully consumed within 2-3 hours of reaction (data not shown). It was epoxidized at rates lower (2-7 fold) than *trans*-2-butene, whereas a 1.5 fold increase in activity was seen with isolate M93A (fig. 43, table 27), suggesting a similar active site configuration for both substrates. Steric factors and angular strain in the substrate as well as the product are the most likely explanations for the low epoxidation rates obtained with cyclohexene (Anderson & Roberts, 1970).

3.5.3. Styrene and phenyl allyl ether

These compounds were epoxidized at similar or lower rates than 1-butene, except with *Staphylococcus* sp. M97B where a 2-4 fold increase in activity was observed (figs. 35, 36 & 44, table 27).

*
A comparison of substrate specificities of microbial epoxidations toward a range of olefins (internal, terminal, cyclic, aryl-substituted), showed that the group of six organisms (M26, M90C, M93A, M142, M156, M186) had a similar reactivity pattern that could not be explained by chemical reactivity differences. On the other hand, the nature of the substrate (electronic effects) appeared to modulate enzymatic epoxidations in *Staphylococcus* sp. M97B. The product was slowly metabolized by an epoxide-degrading enzyme(s) with an apparently 'broad' substrate specificity. Further oxidation was evident on prolonged incubations with olefins (figs. 41, 43 & 44). Non-enzymatic hydrolysis of the product by heat-killed cells was negligible in the standard assay. *Aerococcus* sp. M186 and isolate M156 (capable of growth on alkanes and alkenes) accumulated epoxyalkanes, but were only able to degrade the product at a much slower rate than was found with the other organisms. This may be due to the possibility that the epoxide-degrading enzyme(s) in M186 and M156 is more specific.

Table 27. A comparison of substrate specificities of various alkene-utilizing bacteria.

Bacterial strains	1,2-Epoxybutane				<i>trans</i> -2,3-Epoxybutane				1,2-Epoxyoctane				Cyclohexene oxide				Styrene oxide				Phenyl glycidyl ether			
			Y _{P/S}				Y _{P/S}				Y _{P/S}				Y _{P/S}				Y _{P/S}				Y _{P/S}	
	mole %		mole %		mole %		mole %		mole %		mole %		mole %		mole %		mole %		mole %		mole %		mole %	
	SA	SP	<i>f</i>	<i>c</i>	SA	SP	<i>f</i>	<i>c</i>	SA	SP	<i>f</i>	<i>c</i>	SA	SP	<i>f</i>	<i>c</i>	SA	SP	<i>f</i>	<i>c</i>	SA	SP	<i>f</i>	<i>c</i>
Group-difficult genera M26	18	275	19	ND	7	107	8	ND	3	46	6	7	1	17	3	4	5	77	15	17	21	321	80	89
<i>Micrococcus</i> sp. M90C	51	601	96	ND	7	86	11	ND	8	94	23	23	2	25	7	8	38	445	90	91	18	214	80	89
Group-difficult genera M93A	26	350	22	ND	2	27	4	ND	5	67	7	8	3	40	2	3	6	81	20	21	24	323	85	85
<i>Staphylococcus</i> sp. M97B	5	84	16	ND	9	152	56	ND	5	84	9	11	1	22	3	3	8	135	40	41	19	318	70	74
M142	10	254	20	ND	3	76	9	ND	2	51	5	6	1	25	2	3	4	101	12	12	11	274	38	60
M156	11	203	17	ND	4	74	13	ND	4	74	8	9	1	19	2	3	5	92	24	25	10	184	23	44
<i>Aerococcus</i> sp. M186	13	279	21	ND	5	107	19	ND	5	107	8	9	2	43	3	3	4	85	22	24	9	192	45	56

SA specific activity (nmole/min./mg protein)

SP specific productivity (μmole/hr./g. DW)

Y_{P/S} Bioconversion yield is based on the amount of substrate *fed* (*f*) to or *consumed* (*c*) by each organism.
Y_{P/S} values for gaseous olefins are determined at 10-12th hour of reaction, where epoxide concentration may have not necessarily reached a maximum.

ND not determined, due to practical difficulties involved in accurately calculating microbial gas consumption.

Growth: 4-5 day cultivation, batch
5-10% (V/V) ethene or propene.

Reaction: 25 mM potassium phosphate buffer, pH=7.0 at 30°C. All incubations were done in triplicate.
[Gaseous olefins]=10-40% (V/V)
[Liquid olefins]=2 mM

Total protein 0.35-0.74 mg/ml
Dry weight (DW) 1-3 g/l

Figure 40. Epoxidation of 1-butene by ethene- and propene-utilizing bacteria.

Figure 41. Epoxidation of 1-octene by ethene- and propene-utilizing bacteria.

Figure 42. Epoxidation of *trans*-2-butene by ethene- and propene-utilizing bacteria.

Figure 43. Epoxidation of cyclohexene by ethene- and propene-utilizing bacteria.

Samples were extracted into ether and analyzed by glc
in triplicate.
[1-Styrene]=2 mM

Figure 44. Epoxidation of styrene by ethene- and propene-utilizing bacteria.

3.5.4. 1-Trifluoromethyl, 1-(4' ethoxy phenyl)-ethene

Ethene- and propene-utilizers were also screened for the formation of epoxide from a trifluorinated styrene-based olefin (fig. 45) as part of a research programme carried out at Imperial College (4/7/88 to 30/9/88) sponsored by Imperial Chemical Industries PLC (ICI).

Despite the ability of these organisms to epoxidize styrene and phenyl allyl ether, none were active on ICI's compound. Procedural modifications (various [1-5 mM] substrate concentration, temperature, addition of co-substrates) were, however, unsuccessful. Given the similarities in structure of the substrates, the lack of epoxide formation was surprising. As a possible explanation it is proposed that the electronegativity of the CF_3 substituent would reduce the electron density of the adjacent double bond making it less reactive, although steric effects may also be involved.

Figure 45. 1-Trifluoromethyl, 1-(4' ethoxy phenyl)-ethene.
Its epoxide is a precursor of an agrochemical biocide
(ICI's internal documents).

3.6.

Summary

An extensive isolation programme for organisms capable of growth on olefins was carried out using selective culture techniques. Over 100 bacterial strains (mesophilic, thermophilic) were isolated in pure culture with gaseous or liquid (internal & terminal) olefins as the sole source of carbon and energy. The initial isolation procedures were not straightforward, but required very careful microbiological techniques and a lot of patience. Long periods (5-7 weeks) were required for growth of genuine alkene-utilizers in the culture medium. This is consistent with similar findings that alkene-grown bacteria, in general, have low growth rates with long (5-32 hr.) doubling times. The lag phase indicates that the olefin induces its uptake.

Identification of a selection of organisms, on all seven substrates, revealed the existence of a wide range of genera (table 28), some with unusual biochemical properties that did not fall readily into the genera described in Bergey's Manual of Systematic Bacteriology. There are no reports to date on bacterial isolations on cyclohexene; 3,3-dimethyl 1-butene; 4,4-dimethyl 1-pentene; 2,3-dimethyl 2-butene and allyl cyclopentane. Only a few genera, with a limited substrate specificity, have been isolated on gaseous olefins. These include *Mycobacterium*, *Nocardia* and *Xanthobacter* spp. (table 29) and a Gram-negative motile rod (strain PL-1). Propene was, however, not oxidized in the latter *via* an epoxidation step. The ability to isolate various Gram-negative organisms, has therefore prompted several programmes in molecular biology at Imperial College.

The initial screening involved testing a collection of strains for i) growth rate on the substrate of isolation and ii) evidence of epoxide formation. This resulted in the selection of 18 ethene- and propene-utilizing bacteria that grew well in BMS medium and accumulated epoxyalkanes from terminal (C_2 - C_4) olefins. The pattern of hydrocarbon utilization indicated the presence of two groups of organisms: bacteria that grew a) only on gaseous olefins or b) on both gaseous alkanes and alkenes.

Table 28. Summary of the newly isolated bacterial genera on gaseous and liquid olefins.

Ethene	
<i>Aeromonas</i> sp.	<i>Micrococcus</i> sp.
<i>Aerococcus</i> sp.	<i>Moraxella</i> sp.
<i>Alcaligenes</i> sp.	<i>Staphylococcus</i> sp.
<i>Pseudomonas</i> sp.	GDG
Gram -/+V mesophilic/thermophilic rods and coccobacilli not yet identified.	

Propene	
GDG	
Gram +/V thermophilic rods not yet identified.	

Cyclohexene	
<i>Streptomyces</i> spp.	

3,3-Dimethyl 1-butene	
<i>Micrococcus</i> sp.	<i>Streptomyces</i> spp.
<i>Nocardia</i> spp.	<i>Vibrio</i> sp.
GDG	

4,4-Dimethyl 1-pentene	
<i>Flavobacterium</i> sp.	<i>Nocardia</i> sp.
<i>Pseudomonas</i> spp.	<i>Streptomyces</i> sp.
GDG	

2,3-Dimethyl 2-butene	
<i>Streptomyces</i> spp.	

Allyl cyclopentane	
<i>Alcaligenes</i> sp.	<i>Micrococcus</i> spp.
GDG	

-/+/V indicates a Gram-negative, Gram-positive or Gram-variable reaction

GDG group-difficult genera

Table 29. Reported isolation of bacterial genera on gaseous olefins.

Organism	Isolation substrate	Reference
<i>Mycobacterium</i> spp.	Ethene	de Bont 1976
	Propene	de Bont <i>et al.</i> 1980
	Ethene	Drozd & Bailey 1984
	Butene	Habets-Crützen <i>et al.</i> 1984
	Ethene/Propene/Butene	Fujii <i>et al.</i> 1985
	Vinyl chloride	Hartmans <i>et al.</i> 1985
	<i>trans</i> -2-Butene	van Ginkel <i>et al.</i> 1987b
<i>Nocardia</i> spp.	1,3-Butadiene	Watkinson & Somerville 1976
	Propene	Furuhashi <i>et al.</i> 1981
	1-Butene 1,3-Butadiene	Habets-Crützen <i>et al.</i> 1984
	Isoprene 1,3-Butadiene	van Ginkel <i>et al.</i> 1987a
	<i>trans</i> -2-Butene	van Ginkel <i>et al.</i> 1987c
<i>Xanthobacter</i> spp.	Propene	van Ginkel & de Bont 1986
Strain PL-1 (Gram-negative motile rod)	Propene	Cerniglia <i>et al.</i> 1976

A *Micrococcus* sp. M90C was used to investigate its potential as a biocatalyst for production of chiral epoxides. Ethene-grown cells catalyzed the stereospecific formation of *R*-1,2-epoxypropane (ee=96%), *R*-1,2-epoxybutane (ee=94%), *trans*-(2*R*,3*R*)-epoxybutane (ee=84%), *S*-(+)-phenyl glycidyl ether (ee=93%) and *R*-(+)-methyl phenyl sulphoxide (ee>98%).

Biological resolution of racemic epoxyalkanes *via* enantioselective destruction of one stereoisomer is an alternative method to obtain optically pure epoxides. The bacterium contains an epoxide-degrading enzyme that catalyzes the stereoselective hydrolysis of the (2*S*,3*S*)-isomer of racemic *trans*-2,3-epoxybutane, whereas the (2*R*,3*R*)-isomer is unaffected. Further metabolism of 1,2-epoxyalkanes (C₃-C₄), however, occurs *via* complete destruction of both stereoisomers.

Epoxidation reactions catalyzed by *Micrococcus* sp. M90C exhibited a substrate specificity, different from that expected on the basis of chemical reactivity pattern in peracid epoxidations. Internal (*cis*-/ *trans*-2-butenes), terminal (C₂-C₁₆), cyclic (cyclopentene, cyclohexene) and aryl-substituted (styrene, phenyl allyl ether) olefins are epoxidized as the sole oxidation product, with specific production rates (non-optimized) in the range 0.6-785 $\mu\text{mole/hr./g. DW}$. Studies with a series of straight chain substrates indicated that the reactivity of a terminal double bond toward epoxidation is affected by the length of the alkyl chain. The reactivity was maximal for short chain olefins (C₂-C₄), but decreased markedly as the chain was lengthened (C₈-C₁₆). *trans*-2-Butene was epoxidized more rapidly than *cis*-2-butene. Cyclohexene and *cis*-2-butene were, however, oxidized at comparable rates. Similar production rates were also obtained with propene, cyclopentene and styrene. Factors other than simple chemical reactivity differences are, therefore, being reflected in the enzymatic reactions.

The specificity in *Micrococcus* M90C appears to be modulated by some element of enzymatic control. This can be achieved in two ways: steric constraints on i) substrate interaction in the active site and ii) product formation. The nature of the 'active oxygen' species should, however, not be ignored. An additional level of selectivity is also evident,

i.e., olefins are only epoxidized but not hydroxylated, and the unactivated C-H bonds of alkanes are not attacked. Hence it is possible that the form of 'active oxygen' found in alkene monooxygenases is less reactive than found in alkane monooxygenases. Production of *R*-epoxyalkanes with a very high degree of stereospecificity by *Micrococcus* sp. M90C, also demonstrates that formation of optically active epoxides requires binding of the substrate in such a way as to preclude oxygen attack from one face of the double bond. Steric and electronic factors in the substrate can also affect regioselectivity.

An ethene monooxygenase (EMO) has been implicated in the production of chiral epoxides in cell-free extracts of *Micrococcus* sp. M90C. The enzyme is present in two forms. The soluble EMO, constitutes 32% of the total whole cell activity, requires molecular oxygen and is NADH-dependent. The other form of the enzyme appears to be membrane-bound, comprises only 3% of the total whole cell activity and does not require NADH as an electron donor.

It is known that some methanotrophs may contain either a soluble or a particulate form of the methane monooxygenase. In *Methylococcus capsulatus* (Bath), location of the monooxygenase is determined by the growth conditions, and in particular by the availability of copper ions to the cells. There are, however, no reports on the involvement of a particulate form of the alkene monooxygenase (AMO) in alkene-utilizers. Instability, together with a low monooxygenase activity has precluded purification to date (de Bont *et al.*, 1979; Habets-Crützen *et al.*, 1984). Recently, partial purification of the components of AMO from *Mycobacterium* E3 was attempted. The system was found to be unstable suffering from a very low activity (0.18-3 nmole/min./mg protein) for the production of 1,2-epoxypropane by a mutant of E3 (Hartmans *et al.*, 1989). Demonstration of a stable cell-free activity in *Micrococcus* sp. M90C with a relatively high production rate (19 nmol/min./mg protein) for epoxyethane, has therefore set the basis of future work on the enzymology of EMO in order to elucidate the nature of the enzyme. This aspect is currently under investigation.

Substrate specificity of the majority of gaseous alkene-utilizing bacteria reported to date is restricted to a rather narrow range of olefins despite the stereospecificity of the

epoxidation reactions. These organisms also contain an epoxide-degrading enzyme(s) that often hydrolyzes the product at a faster rate than the oxidative step, thus preventing any significant accumulation of epoxyalkanes. Susceptibility of the organisms toward epoxides has also been suggested as a plausible explanation for the lack of product accumulation. Ethene-utilizing *Micrococcus* sp. M90C, however, contains an epoxide-degrading enzyme(s) that *slowly* metabolizes the product, resulting in the accumulation of epoxyalkanes. The ability to catalyze asymmetric sulphur oxygenation as well as epoxidation of a wide range of olefins in very high optical and chemical yields, demonstrates its potential as a 'general purpose' biocatalyst for the production of optically pure oxygenated compounds in a biotechnological process.

In search of better biocatalysts, the remaining 17 ethene- and propene-utilizing bacteria were screened for the ability to i) form optically active epoxides from lower gaseous olefins and ii) epoxidize phenyl allyl ether. All of the strains, stereoselectively formed *R*-1,2-epoxypropane (ee=90-96%), *R*-1,2-epoxybutane (ee=90-98%) and *trans*-(2*R*,3*R*)-epoxybutane (ee=64-88%). The majority of the organisms (14 strains), accumulated phenyl glycidyl ether as the sole product of phenyl allyl ether oxidation with specific production rates (non-optimized) in the range 69-323 $\mu\text{mole/hr./g. DW}$. The two ethene-utilizing bacteria (M26, M93A) each catalyzed the stereospecific formation of *S*-(+)-phenyl glycidyl ether (ee=93%). This has attracted a considerable industrial interest and is currently under investigation at ICI for commercial production of optically active β -blockers.

In addition to *Micrococcus* sp. M90C, the substrate specificities of 6 other organisms were studied in order to probe the active site of the enzymes involved. The pattern of reactivity for the group of 4 ethene- (M26, M90C, M93A, M186) and 2 propene- (M142, M156) utilizers differed from that expected with peracids, suggesting a considerable degree of enzymatic control. The nature of the substrate, however, modulated enzymatic epoxidations in *Staphylococcus* sp. M97B.

After many years of study, Foster (1962) reached the conclusion that the types of microorganisms isolated from hydrocarbon enrichment cultures, were 'unexpectedly restricted and furthermore always had the same complexation'. His views are summarized in The Second A.J. Kluyver Memorial Lecture (Foster, 1962) where it is documented: ' . . . that the predominant microorganisms that emerge as pure culture isolates from hydrocarbon enrichments or selective cultures, were more or less taxonomically related belonging to the genera *Brevibacterium*, *Corynebacterium*, *Mycobacterium* and *Nocardia* . . . thus, if we are to generalize, we would have to conclude that the above-named groups are those which in nature do the lion's share of the attack on hydrocarbons . . . '

Given the success of isolating many different genera of alkene-utilizing bacteria, it would suggest that the ability to utilize olefin hydrocarbons is more widespread in nature than previously recognized.

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