Development of a Process for the Production of Propylene Oxide in Methane-oxidising Bacteria

by

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DEDICATION

To my Mother, Father and Kuniko.

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Summary

The aim of this project is to develop a process for the production of propylene oxide (PO), using methane-oxidising bacteria. At the beginning, a difficult problem needed to be solved for the development of this process i.e. the short life-span of the biocatalyst. Experiments showed that the cells of the methane-oxidising bacterium, Methylococcus capsulatus (Bath) lost their catalytic activity within 30 minutes under the conditions of high PO production. The inactivation of the biocatalyst was largely independent of externally-accumulated PO but was totally dependent on PO produced in vivo under conditions of high PO production. The cells lost their activity without any external accumulation of PO under those conditions where PO was produced. Prior to the research of the present writer, it had been concluded that external PO inactivated the biocatalyst. A specific PO productivity of more than 700 nmol PO produced/min/mg cells was obtained in the work reported here. However, by increasing the PO productivity more than 200 mU/mg cells, the cells lost their activity rapidly and their half-life lasted 7 minutes.

In order to overcome the short life-span of the biocatalyst, a reactivation of the inactivated cells had to be devised. The methane-oxidising bacteria contain an enzyme, methane monooxygenase (MMO) which oxidises methane to methanol and also oxidises propylene to PO. The MMO was irreversibly inactivated by acetylene or by PO, however this inactivated MMO was reactivated by subjecting the cells to reactivation treatment. This reactivation process is a phenomenon not previously known about. In order to reactivate the inactivated MMO, the cells required carbon, nitrogen and sulphur sources. In addition, a suitable oxygen and temperature regime was required for the reactivation process. The requirement of nutrients for reactivation and the inhibition of reactivation by the addition of chloramphenicol led to the conclusion that protein synthesis was associated with the reactivation process. Furthermore, it was found that MMO synthesis was completely inhibited by a detectable amount of methanol in the cell suspensions. Copper was not required for the reactivation of cells which contained particulate MMO.

Two types of inactivation mechanism were assumed under the conditions of PO production. These are the inactivation of MMO and the inactivation of the biocatalyst by a means not yet identified. When the MMO only was inactivated, it was reactivated quickly. However when these cells were inactivated under conditions of high PO production, they required three times as long a period for complete reactivation than did those cells which had been inactivated by acetylene. This delay in the reactivation process was thought to be due to a concealed inactivation (unidentified inactivation) factor. The latter was thought to be caused by the accumulation of PO within the cells. The intracellular PO concentration was calculated on the basis of the retention time of PO in the cells, and its concentration appeared to be related to PO productivity. The concealed inactivation was assumed to be due to a solvent-like effect of PO in the cells and not from an alkylation effect.

In order to develop a PO production process using the reactivation system, Methylocystis parvus (OBBP) was selected as the best organism from 25 methanotrophs. The reactivation system, the growing-cell process (single stage) and the two-stage reactivation process were designed and operated. Using the growing-cell process, continuous PO production was achieved at a rate of 12 g/l/day for a period of more than three weeks.

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CHAPTER 1

General Introduction

1.1 The Concept of Methylotrophy

In 1972, Colby and Zatman proposed and defined the term 'methylotrophy'. According to their definition, methylotrophs are organisms capable of obtaining energy by the oxidation of C_1-growth substrates and the assimilation of carbon such as formaldehyde or as a mixture of formaldehyde and carbon dioxide, but always by pathways that are distinguishable from the Calvin cycle. This group of organisms can be further sub-divided into two groups:- obligate and facultative methylotrophs, the latter group having the additional capability to grow and replicate on a variety of other carbon-to-carbon bond compounds.

Methylotrophs, as defined above, are organisms which can utilize methane, methanol, N-methyl compounds, S-methyl compounds as their sole sources of carbon for growth and replication. The extensive and varied physiology and biochemistry of all the different types of methylotrophs have been comprehensively written about in a number of reviews by Quayle, 1972, Colby et al., 1979; Higgins et al., 1981a; Anthony, 1982; Large and Bamforth, 1988. The reader, therefore, should use these sources of reference for more detailed information on methylotrophy.

1.2 Occurrence , Ecology and History of the Isolation of Methane-oxidising Bacteria

The extent of turnover of methane within the biosphere as the major carbon source of methane-oxidising bacteria (methanotroph) is not widely appreciated. In 1976, Ehhalt reported approximately 50% of all organic carbon, decomposed by anaerobic microflora is converted into methane. The methane amounts to 5.3 - 8.1 mega tonnes per year.

Biogenic methane is the major source of atmospheric methane and is quantitatively similar to that of the output from natural gas wells.

The disparity between the amount of methane in the atmosphere and methane generated by methanogenic bacteria is due mainly to the activities of methane-oxidising bacteria which are widely distributed in the environment, including the possibility that some methane may be oxidised anaerobically (Pangariban et al., 1979; Raeburgh, 1981).

Although methane-oxidising bacteria are now known to be widely distributed in nature, prior to 1970, only three species of methane-oxidising bacteria had been isolated and characterized (Foster and Davies, 1966; Brown et al., 1964, Stocks and McCleskey, 1964) despite the fact that the first isolate had been found early in this century by Söhngen (1906). The difficulty of isolating pure cultures, particularly of methane-oxidising bacteria was certainly due to the lack of a reliable enrichment and isolation technique. When enriched cultures were plated on agar plates under an atmosphere of methane and air, many scavengers formed colonies which, when replated on fresh agar plates, formed further colonies even without methane.

The other problem encountered was symbiosis. It was sometimes found that methane-oxidising bacteria grew well in a mixed culture (Linton and Buckee, 1977. Imai et al., 1986). The enrichment and isolation technique was dramatically transformed in 1969-1970 by Whittenbury and his colleagues. They devised simple and effective techniques for the isolation of methane-oxidising bacteria (Whittenbury, 1969; Whittenbury et al., 1970b) from which they isolated more than a hundred different strains. The success of their techniques derived from the employment of a shorter enrichment period. This limited losses of bacteria experienced as a result of predation and overgrowth growing on substrates other than methane.

Whittenbury et al. (1970a) classified their isolates into five groups; Methylococcus, Methylomonas, Methylobacter, Methylosinus and Methylocystis. The importance of the studies undertaken in the isolation

technique is evident from the widespread and varied research into the isolation of methane-oxidising bacteria (Quayle, 1972).

1.3 Classification of Methane-oxidising Bacteria

Confusion relating to the nomenclature and taxonomy of methane-oxidising bacteria has not yet been conclusively resolved, however the best scheme produced to date is Whittenbury et al. (1970a) who found a correlation between the type of membrane arrangement, and the means employed for carbon assimilation. Type I organisms assimilate carbon by the ribulose monophosphate pathway, whereas organisms possessing Type II membrane systems used the serine pathway.

Table 1.3.1 shows the classification of obligate methane-oxidising bacteria proposed by the above authors. However, since the adoption of this scheme of classification a number of reports have appeared with suggest that the two groups are not as clearly defined as was first thought. It has been shown that some of Type I and II methanotrophs can possess both hexulose phosphate synthase and hydroxypyruvate reductase activity and this is indicative of the presence of the ribulose monophosphate cycle and the serine pathway respectively which has led to the proposal of the third group of methanotrophs, the Type X group (Whittenbury and Dalton, 1981).

The criteria used to resolve the problem of classification of methanotrophs are, at present, unsatisfactory since the nature of the organisms makes this task difficult. In 1984, Omelchenko and Andreev devised a scheme for the classification of methanotrophs which was based on certain characteristics including:-

Table 1.3.1

Classification to Obligate Methane-utilising Bacteria

Character	Type I	Type II
Membrane arrangement	Bundles of vesicular discs	Paired membranes in layers around periphery
Resting stage	Cysts (Azotobacter-like)	Exospores or "lipid-cysts"
Carbon Assimilation	Ribulose monophosphate pathway	Serine pathway
TCA cycle	Incomplete (lacks 2-oxoglutarate dehydrogenase)	Complete
Glucose-6-phosphate and 6-phosphogluconate dehydrogenase	Present	Present
Examples	Methylococcus Methylomonas Methylobacter	Methylosinus Methylocystis Methylobacterium

phospholipid, DNA homology and protein composition. Their classification produced results which coincided with that of an earlier scheme devised by Whittenbury et al. 1970b).

It is of paramount importance that the problem relating to correct classification of methanotrophs is thoroughly resolved, not just because it would give academic satisfaction, but because of the implications for industries. For example, in both Europe and Japan, microbial patents are issued according to genus and/or species these can cover all the species which belong to that genus. Even if new species are found, these species are restricted by the patent which has been already issued. But the patent has no restriction on any other genus. The confusion of classification makes it easy to propose a new genus. In this case a patent which has been issued, has not restriction for the newly proposed genus. In the United States, patents are issued according to species only, so the above problem does not arise.

1.4 Physiology and Biochemistry of Obligate Methane-oxidising Bacteria

1.4.1 Basic Growth Requirements

Methane-oxidising bacteria are strictly aerobic, due to their need for gaseous oxygen in the initial oxidation of methane (Higgins and Quayle, 1970). They are capable of utilising either methane or methanol as a sole source of carbon and energy. The growth of methane-oxidising bacteria is inhibited by the addition of ordinary heterotrophic metabolites in normal concentrations (Eccleston and Kelly, 1972, 1973). No growth factors are required for the growth of these organisms as they are normally grown on a mineral-salts medium, containing a nitrogen

source, calcium, magnesium, potassium, sulphate, phosphate and trace elements (Dalton and Whittenbury, 1976). Some amino acids stimulate the growth of methane-oxidising bacteria (Murrell, 1981).

1.4.2 Carbon Metabolism

Methane-oxidising bacteria are capable of oxidising methane to carbon dioxide completely. Methane is first of all oxidised to methanol by way of the action of a methane monooxygenase (MMO). Methanol is then further oxidised to formaldehyde by a methanol dehydrogenase. Formaldehyde can be assimilated into the cell to form cell materials or it can be further oxidised by way of a dissimilatory route via formate and finally to carbon dioxide, to provide the cell with energy for its assimilatory pathway. Both assimilatory and dissimilatory pathways work simultaneously in the cell.

1.4.3 C1-compound Assimilation Pathways

Three devices for the assimilation of C_1-compounds are recognized to date:- the ribulose monophosphate pathway for formaldehyde assimilation, the serine pathway and the ribulose diphosphate pathway (Calvin cycle) for carbon dioxide assimilation. Some organisms may use more than one mechanism for C_1-assimilation either when subjected to different growth conditions; or indeed when two mechanisms are used simultaneously. However there is no convincing evidence as yet of two or more complete C_1-assimilation pathways operating simultaneously in any one micro-organism. Nevertheless, it is clear that certain C_1-utilizers, while using one pathway as the major source of fixed carbon are capable of assimilating small amounts of C_1-compound by a different pathway (Higgins, 1981).

1.4.4 Ribulose Monophosphate Pathway (RMP)

Ribulose monophosphate pathway (RMP), a pathway of formaldehyde assimilation was initially proposed by Kemp and Quayle (1967) and further elaborated by Lawrence and Quayle (1970a) and Kemp (1974). The overall effect of the cycle is to synthesize a C_3-compound from three molecules of formaldehyde. The cycle is conveniently divided into three stages; fixation, cleavage and rearrangement.

Stage 1, Fixation: by the action of hexulose phosphate synthase, three molecules of formaldehyde are condensed with three molecules of ribulose-5-phosphate to yield three molecules of fructose-6-phosphate.

Stage 2, Cleavage: one molecule of hexulose-6-phosphate is isomerised to fructose-6-phosphate and this is then split into two C_3-compounds. This being achieved either by the enzymes of the glycolytic sequence or by the Entner-Doudoroff pathway enzymes.

Stage 3, Rearrangement: this stage involves the regeneration of the three molecules of ribulose-5-phosphate from the two molecules of fructose-6-phosphate and one molecule of glyceraldehyde-3-phosphate produced in stages 1 and 2.

1.4.5 Serine Pathway

The carbon assimilation pathway in the Type II methane-oxidising bacteria were first studied by Quayle and co-workers (Lawrence et al., 1970a, 1970b). The overall result of the pathway is to incorporate two molecules of formaldehyde and one molecule of carbon dioxide into a C_3-compound, 3-phosphoglycerate. The cycle is initiated by two molecules of serine which are formed from two molecules of glycine plus two molecules of $N^{5,10}$ methylenetetrahydrofolate by the action of serine

transhydroxymethylase. By a series of reactions, serine is converted into 3-phosphoglycerate for assimilation into cell carbon or converted into phosphoenol pyruvate (PEP). PEP carboxylase catalyses the carboxylation of PEP with carbon dioxide to form oxaloacetate, which subsequently forms malyl-CoA. The malyl-CoA is then cleaved into two C_2 units to act as further acceptors of C_1 units to maintain the cycle of reactions.

1.4.6 Ribulose Diphosphate Pathway of Carbon Dioxide Assimilation

Methylococcus capsulatus (Bath), a Type I methanotroph, appeared to use only the ribulose monophosphate pathway for C_1 assimilation (Strøm et al., 1974). However, it has now been shown to possess hydroxypyruvate reductase, a key enzyme in the serine pathway, although at low level (Read, 1976). Furthermore the presence of the key enzymes of the Calvin cycle, ribulose diphosphate carboxylase and phosphoribulokinase were demonstrated in cell extracts of Methylococcus capsulatus (Bath) (Taylor, 1977). The rate of CO_2 fixation by whole cell was low, and contributed only about 2.5% (wt/wt) of the total cell carbon. Fixation was observed only with the presence of methane, indicating an energy requirement for incorporation (Taylor et al., 1980).

Ribulose diphosphate carboxylase from this organism requires a divalent cation for activity. It has an alkaline pH optimum; is inhibited by 6-phosphogluconate and possesses an oxygenase activity (Taylor et al.,1980). This secondary activity of the enzyme generates phosphogluconate, which may be subsequently cleared by a specific phosphogluconate phosphatase. This was observed in extracts of Methylococcus capsulatus (Bath) (Taylor et al., 1981). The metabolic fate of the glycolate may possibly involve incorporation by way of

glyoxylate and the serine pathway. This provides a role for this pathway in a Type I strain, which incorporates label from [^{14}C] glycolate into serine and glycine (Taylor et al., 1981).

1.5 Methane Oxidation

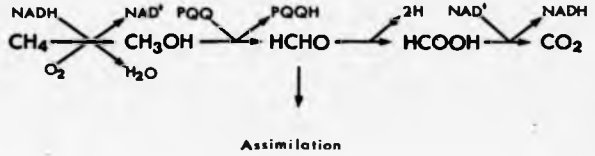
The pathway for the complete oxidation of methane has been generally assumed to involve the following series of reactions, originally proposed by Dworkin and Foster (1956) and has since been refined by several workers (Fig. 1.5.1).

The oxidation of methane to carbon dioxide appears to proceed via a series of two-electron oxidation steps. The first reaction is hydroxylation catalysed by the methane monooxygenase (MMO) which in vitro requires NAD(P)H as a reductant. The second reaction is conversion of methanol to formaldehyde and is catalyzed by a methanol dehydrogenase which contains a novel prothetic group, pyrrolo-quinoline quinone group (PQQ). However in Methylococcus capsulatus the particulate enzyme does appear to have oxidase activity as well (Wadzinski and Ribbons, 1975). Formaldehyde occupies a central position in the metabolism of methane since it is both assimilated into biomass and dissimilated to carbon dioxide to provide energy for growth.

1.5.1 Two Types of Methane Monooxygenase, Soluble- and Particulate MMO

The two types of MMO, soluble MMO and particulate MMO can exist in certain methanotrophs. The existance of these two forms depend on the conditions under which the organism is grown (Scott et al., 1981a,b; Stanley et al., 1983).

Methylosinus trichosporium grown in a chemostat under



PQQ = Pyrrolo-Quinoline Quinone

Fig. 1.5.1 The oxidation of methane by methanotrophs

oxygen-limiting conditions had 100% particulate MMO activity, under nitrate limitation, 100% soluble MMO was observed. Under methane-limiting conditions, a mixture of soluble and particulate MMO's were obtained (Scott et al., 1981a,b). Stanley et al. (1983) showed that the intracellular location of the MMO in Methylococcus capsulatus (Bath) depended on the availability of copper and was not dependent on either methane- or nitrate-limitation. Particulate MMO was observed under conditions with copper in excess, whereas under conditions of copper stress a soluble MMO was found. The two conditions were not mutually exclusive since it was possible to provide environmental conditions under which both forms of the enzyme could be stably maintained.

1.5.2 Soluble Methane Monooxygenase

The soluble MMO obtained from crude cell extracts of Methylococcus capsulatus (Bath) is the best characterized MMO among the methanotrophic organisms studied so far and has been purified to near homogeneity. The early work on the purification system has been reviewed by Dalton (1980).

The soluble MMO from Methylococcus capsulatus (Bath) was found to be a complex of three proteins A,B,C, which could be resolved by DEAE-cellulose chromatography and purified by chromatographic technique (Colby and Dalton, 1978; Dalton, 1980; Woodland and Dalton, 1984 a,b).Protein A which is a 220,000 molecular weight protein, comprised of three subunits; alpha, beta, gamma which have molecular weights of 54,000, 42,000 and 17,000 daltons respectively.

Protein A contains non-heme iron (0.2 - 0.5 atoms per mole) and zinc (0.2 - 0.5 atoms per mole) but no acid labile sulphide. Protein B, a 16,000 molecular weight protein which is acidic but which contains no prosthetic group. Protein C is a single polypeptide protein of molecular

weight 38,000 containing one molecule of flavin adenine dinucleotide (FAD) and one Fe_2 S_2 centre per molecular (Colby and Dalton 1978, 1979; Woodland and Dalton, 1984a; Lund and Dalton, 1985; Lund et al., 1985)

Protein A has no discernible independent catalytic activity. However protein A is believed to be the component responsible for substrate binding. A large change in the electron paramagnetic resonance (EPR) spectrum of reduced protein A is observed in the presence of a substrate (Dalton, 1980, Woodland and Dalton 1984a). Furthermore, Prior and Dalton (1985b) reported that the acetylene, a suicide substrate of MMO in vitro, would associate with the alpha subunit of protein A and inactivated MMO activity.

Of the three soluble MMO components, only protein C has independent catalytic activity. It is an NAD(P)H acceptor reductase which catalyses the NAD(P)H driven reduction of cytochrome c, potassium ferricyanide, dichlorophenol-indophenol or rather poorly, oxygen (Colby and Dalton, 1979). The recent development in the purification procedures for all three proteins has enabled the investigations of the mechanism of methane oxidation by the reconstituted complex. Protein C, as already stated, can transfer electrons from NADH to a wide range of electron accepters including protein A. By the use of EPR spectroscopy, the ordering of the redox couples of the FAD and Fe_2 S_2 redox centres was carried out (Lund and Dalton, 1985). The redox centre can exist in odd electron forms, $0e^{-1}$ (oxidised), $1e^{-1}$ (semiquinone), $2e^{-1}$ (mostly semiquinone and reduced Fe_2 S_2), and $3e^{-1}$ (dihydroquinone and Fe^{2} S^{2}). This ability suggests a role for protein C as a $2e^{-1}/1e^{-1}$ transformase, electron pairs from NADH being split up and donated to protein A as single electrons of equal redox potential. FAD appears to interact with NADH, transferring single electrons onto Fe_2 S_2 which donates them to protein A (Lund and Dalton, 1985, Lund et al., 1985).

Protein C can therefore pass electons singly from NADH to protein A at constant redox potential. These electrons can then be used to reduce oxygen to water resulting in an NADH oxidase activity for the protein A plus C complex (Lund *et al.*, 1985). This occurs in the absence of protein B, and so electron flow is independent of the presence of protein B. Protein B does however act to shut down this electron flow (Green and Dalton, 1985). On the addition of a suitable substrate, electron flow and oxygen uptake are immediately stimulated, the complete monooxygenase complex being active. The addition of substrate to protein A and C has no effect on the flow of electrons. Protein B therefore appears to act to couple the flow of electrons from NADH through protein C to protein A to the oxidation of substrate, switching the enzyme complex from an oxidase to an oxygenase (Green and Dalton, 1985).

Many investigations on the soluble MMO from other methanotrophs have been demonstrated: *Methylosinus trichosporium* (OB3b) (Stirling and Dalton, 1979; Burrows *et al.*, 1984; Pilkington, 1986), obligate methanotroph SB1 (Allen *et al.*, 1984) and *Methylobacterium* sp. CRL-26 (Patel, 1984).

1.5.3 Particulate Methane Monooxygenase

The only membrane-bound MMO which has been purified so far is the enzyme from *Methylosinus trichosporium* (OB3b) (Tonge *et al.*, 1975, 1977). The method used for isolation involved removal of the enzyme from the cell membrane by phospholipase treatment. However, this procedure was later reported to be no longer effective (Higgins *et al.*, 1981a).

Prior (1985) attempted to purify the particulate MMO of *Methylococcus capsulatus* (Bath) using a variety of solubilising agents.

He noted that N-D-glucose-N-methylalkanamide, which had been successfully used in solubilization of membrane bound antibodies from plasma membranes (Hildreth, 1982) appeared to solubilize this enzyme, however, the enzyme was not truly soluble but was associated with small membrane fragments. Prior (1985) did not succeed in solubilizing the particulate MMO, however he did tentatively identify the protein involved with the active site by using radio-labelled acetylene which bound to a 26,000 molecular weight protein in the particulate fraction.

1.6 Methanol Oxidation

The oxidation of methanol by whole cells is quite important for the production of chemicals using MMO as a catalyst since methanol is a cheap and effective electron donor. Methanol oxidation in methylotrophs appears to be mediated by a methanol dehydrogenase, although in Methylococcus capsulatus the particulate enzyme does appear to have oxidase activity as well (Wadzinski and Ribbons, 1975). Methanol oxidation by an NAD-independent dehydrogenase was first described in Pseudomonas M27 by Anthony and Zatman (1964a,b). Subsequently, many reports appeared on both obligate and facultative methylotrophs. These are reviewed by Anthony (1982).

Methanol dehydrogenases, isolated from approximately thirty methylotrophic bacteria (Anthony, 1982) have been characterised and appear to be quite similar. They are usually dimers of identical subunits of 60,000 daltons.

They have an optimum pH of around 9 and they are often stable at pH 4 in vitro. Enzymic activity in cell extracts requires the addition of phenazine methosulphate as an electron accepter and ammonia or methylamine as an activator. The in vivo electron acceptor from methanol dehydrogenase is thought to be cytochrome c. This conclusion

is based on the reduction of cytochrome c by methanol dehydrogenase (Duine et al., 1979; O'Keefe and Anthony, 1980). However, in some methanotrophs, methanol dehydrogenase may directly couple with particulate MMO (see Chapter 3).

In general, the substrate specificity of methanol dehydrogenase is wide (Anthony and Zatman, 1965). Methanol dehydrogenase activity, which is sometimes called primary alcohol dehydrogenase, is restricted to primary alcohols with the affinity of the enzyme decreasing with increasing carbon chain length, and the three dimensional configuration is more important in determining whether or not they are oxidised. Thus 1,2-propane diol, having two substituents on its second carbon atom, is not oxidised by most of the methanol dehydrogenases studied, however Bolbot and Anthony (1980a) reported the novel methanol dehydrogenase isolated from Pseudomonas AM1 that oxidises 1,2-propane diol.

The common characteristic of all methanol dehydrogenases tested was their ability to catalyse the oxidation of formaldehyde to formic acid. The rate of formaldehyde oxidation was usually similar to that for methanol oxidation and the affinity of the enzyme for the two substrates was often found to be similar. It has been suggested that the actual substrate during formaldehyde oxidation was the hydrated aldehyde (diol) and that the extent to which other aldehydes were oxidised might be related to their degree of hydration (Sperl et al., 1974). One unusual methanol dehydrogenase, derived from Rhodopseudomonas, also catalysed the oxidation of formaldehyde, acetaldehyde and propion aldehyde, although rates were similar to those measured for ethanol (Sahm et al., 1976; Beenforth and Quayle, 1978b).

Anthony and Zatman (1967) described the in vitro fluorescence characteristics of methanol dehydrogenase and showed that it possessed a novel prosthetic group which is a feature of all methanol dehydrogenases. The structure of this prosthetic group was determined

by Duine et al. (1980) and was named "pyrrolo-quinoline quinone (PQQ)". Recently, a novel NAD-dependant methanol dehydrogenase has been isolated by Duine et al. (1984a). This enzyme still contains the prosthetic group PQQ, but is tightly bound to NADH dehydrogenase. This type of methanol dehydrogenase was isolated from Nocardia sp. 239 and Methylococcus capsulatus (Bath) (Duine et al., 1984b). The implications of this discovery of NAD-linked dehydrogenase in Methylococcus capsulatus (bath) are important as this enzyme could possibly provide reducing power for MMO systems in the form of NADH.

1.7 Growth on Methanol and MMO Activity

Many reports have appeared in the literature concerning the growth of methanotrophs on methanol as their sole source of carbon and energy. Early reports suggested that methanol was toxic to the methanotrophs even at concentration as low as 0.01% (v/v), and growth was poor (Leadbetter and Foster, 1958; Stocks and McCleskey, 1964; Whittenbury et al., 1970b). The reason for this poor growth was not known, but Ribbons et al. (1970) suggest that growth may be inhibited by the accumulation of formaldehyde in the methanol-grown cultures. Formaldehyde inhibition of whole cells has been reported in Methylomonas sp. BC3 at 0.015% (Chan et al., 1977) and in Methylocystis parvus (OBBP) at 0.012% (v/v) (Hou et al., 1979a). Actually, accumulation of formaldehyde in the medium of shake-flask grown cultures of Methylococcus NCIB 11083 was demonstrated by Linton and Vokes (1978).

Methanol is a very attractive carbon source for the cultivation of methanotrophs from the point of view of handling, safety and solubility in water compared with methane, assuming that methanotrophs can grow well on methanol and have a high MMO activity. Growth of Methylocystis parvus (OBBP) on methanol can be achieved by using low concentrations of

methanol in the starter culture medium and slowly increasing the concentration over successive transfers to fresh medium so that cells can become "adapted" to growth on methanol at a concentration of up to 4% (Hou et al., 1979a). Growth on methanol can also be achieved by growing the organism in chemostat culture under methanol limitation in which the methanol concentration in the in-flowing medium was 0.25% (v/v) but the concentration in the vessel was ostensibly zero (Linton and Vokes, 1978; Best and Higgins, 1981).

It has long been a controversy whether or not MMO activity was present in cells which had been grown on methanol as a carbon source. Hou et al. (1979a) claimed that growth of Methylosinus trichosporium (OB3b), Methylococcus capsulatus CRL - M1 and Methylobacterium organophilum CRL - 26 on methanol caused the loss of MMO activity and they concluded that MMO was induced by methane. Other reports of growth of methanotrophs on methanol (Linton and Vokes, 1978, Hyder et al., 1979; Chatina and Trotsenko, 1981) have shown that the MMO activity was retained even when cells were grown on methanol for periods of up to 9 months (Best and Higgins, 1981). Recently, Stanley et al. (1983) and Prior and Dalton (1985a) have demonstrated that copper causes the switch between soluble and particulate MMO synthesis such that at low copper concentrations, soluble MMO is sythesized while at higher copper concentrations the particulate MMO is synthesized. However, cells grown on methanol appear to synthesise only the particulate MMO in response copper concentration. Very low soluble MMO can be detected in the methanol grown cultures when the copper concentration was low.

1.8 Secondary Alcohol Oxidation

It has been known that n-alkanes are oxidised to methylketones by Methylomonas methanica (Leadbetter and Foster, 1960., Dworkin and

Foster, 1956). Methanol dehydrogenases from methane-oxidising bacteria have a wide substrate specificity for C_1 to C_{10} primary alcohols, but shows either little or no activity for secondary alcohols. However, a secondary alcohol-specific dehydrogenase activity was observed in cell extracts and whole cell suspensions of several types of obligate and facultative methane- and methanol-oxidising bacteria (Hou et al., 1979a; Wolf and Hanson, 1978; Goldberg, 1976; Bellion and Wu, 1978).

MMO produces both primary and secondary alcohols from short chain n-alkanes (Colby et al., 1977; Patel et al., 1980; Higgins et al., 1980). Hou and co-workers described the accumulation of methylketones from secondary alcohols (Hou et al., 1979a) and from n-alkanes (Hou et al., 1981a; Patel et al., 1980) using whole cells. Lynch et al. (1980) reported that activity could not be demonstrated in extracts of either of the facultative methanotrophs Methylobacterium ethanolicum or Methylobacterium hypolymeticum, although whole cells oxidised 2-butanol. The activities of secondary alcohol dehydrogenase in methanotrophs are relatively low compared to the methanol dehydrogenase activities. Hou et al. (1979c) does not ascribe a physiological function to the secondary alcohol dehydrogenase activity in C_1-metabolism, but does suggest that this NAD^+-linked activity would be advantagious for methanotrophs, which may be NAD(P)H-limited.

1.9 Formaldehyde Oxidation

It has been well known that all the methanol dehydrogenases mentioned before oxidise formaldehyde (Sperl et al., 1974). C_1-utilising bacteria contain several different types of formaldehyde dehydrogenase activity (Stirling and Dalton, 1978). In Methylomonas methanica, there may be three or four mechanisms for oxidising formaldehyde. Although in Methylococcus capsulatus (Texas), methanol

dehydrogenase may be the only mechanism for formaldehyde oxidation. These various types can be roughly classified into two groups:-

1) $NAD(P)^+$-linked enzymes

2) $NAD(P)^+$-independent enzymes

The enzymes in the first group include both formaldehyde-specific and non-specific aldehyde dehydrogenases, and many require glutathione for activity. Stirling and Dalton (1978) purified an $NAD(P)^+$-linked formaldehyde dehydrogenase from Methylococcus capsulatus (Bath) which required the presence of a heat-stable cofactor from cell extracts for activity and subsequently reported preliminary evidence for an NAD^+-linked enzyme in cell extracts of Methylosinus trichosporium (OB3b) (Stirling and Dalton, 1979).

The second group of enzymes all require artificial electron acceptors to assay their activity in vitro, and some can utilize various other aldehydes as substrates. These are reviewed by Stirling and Dalton (1978), Zatman (1981) and Anthony (1982). Formaldehyde can also be oxidised to CO_2 and water by a cyclic series of reactions involving hexulose phosphate synthase (Ström et al., 1974; Colby and Zatman, 1975). Theoretically, this pathway could generate two molecules of NAD(P)H per molecule of formaldehyde oxidised. The ability to derive available reducing equivalents in the form of NAD(P)H from formaldehyde dehydrogenases may be important in influencing the yield during growth on methane (Anthony, 1978). Furthermore, it will influence the energy supply when methane-oxidising bacteria are used as a biocatalyst for production of chemicals.

Recently, Green and Dalton (unpublished data) have isolated a low molecular weight protein from Methylococcus capsulatus (Bath) which is involved in regulating the activity of the enzyme, formaldehyde

dehydrogenase. This protein is of interest to biotransformation studies since it changes the substrate specificity of formaldehyde dehydrogenase. A regulatory protein (M-protein) of methanol dehydrogenase has also been reported in Pseudomonas AM1 (Ford et al., 1985) and Methylophilus methylotrophus (Page and Anthony, 1986), which alters the substrate specificity of that enzyme also.

1.10 Formate Oxidation

Two types of formate dehydrogenase have been described in bacteria; one is a soluble, NAD^+-linked enzyme which is specific for formate and the other is a membrane-bound NAD^+-independent enzyme which donates electrons to the cytochrome chain at the level of cytochrome b (Dijkhuizen et al., 1978, 1979; Rodinov and Zakharova, 1980). Formate is oxidised in methylotrophs by a soluble dehydrogenase which is specific for formate and NAD^+. The distribution and specific activities of formate dehydrogenase in a variety of bacteria are detailed by Zatman (1981). In many methylotrophs, the formate dehydrogenase appears to be the only enzyme providing NADH for biosynthesis during growth on C_1-compounds. It is not essential in those bacteria able to oxidise formaldehyde by the cyclic series of reactions involving hexulose phosphate synthase, but bacteria lacking this enzyme are unlikely to be able to grow on formate.

1.11 The Industrial Applications of Methylotrophs

Anthony (1982) suggested three main areas of commercial exploitation of methylotrophs in his book:-

a) production of single cell protein (SCP)

b) overproduction of metabolites;

c) the use of methylotrophs and their enzymes as biocatalysts.

Large and Bamforth (1988) also reviewed these industrial applications of methylotrophs in their book.

Methane and methanol are viable C_1 carbon sources which can compete with the more traditional carbon sources for the large scale cultivation of micro-organisms. Namely, these carbon sources are more beneficial when product prices are cheap. Methane can, in general, be obtained even more cheaply than methanol, but it is less favourable as a carbon feedstock for the following reasons (Large and Bamforth, 1988):-

a) demands a high oxygen input into cells;

b) danger of the explosive gas mixture;

c) relatively low solubility in water;

d) low growth yield.

On the other hand, big advantages of methanol as a fermentation feedstock are its relative cheapness and slow price increases compared with the wildly fluctuating prices of sucrose (Hacking, 1986; Linton and Niekus, 1987), high purity, ease of handling, etc., (see Large and Bamforth, 1988).

In 1977, Colby et al., showed the notable results about the oxidation of hydrocarbons by methane-oxidising bacteria. The substrate specificity of soluble MMO was such that it oxidised not only alkanes, but also alkenes, aromatic and alicyclic compounds. Following this report, Higgins et al. (1979, 1980) and Hou et al. (1979c, 1980) demonstrated similar findings on the oxidation of hydrocarbons by cell extracts, MMO fractions or whole cell suspensions. The results of studies on the oxidative potential of methanotrophs that have been

published are summarised in Table 1.11.1. The observation that MMO is responsible for the insertion of oxygen into many different organic substrates can be classified into several reaction patterns:-

a) hydroxylation of normal alkanes, cyclic alkanes and aromatic compounds
b) epoxidation of normal and cyclic alkenes
c) dehalogenations
d) N-oxidation
e) O-demethylation.

The soluble MMO of Methylococcus capsulatus (Bath) catalyses the hydroxylation of primary and secondary alkyl C-H bonds, the epoxidation of terminal and internal alkenes. Furthermore, soluble MMO catalyses the oxidation of CO to CO_2, the oxidation of methanol to formaldehyde and the dehalogenation of halogenated C_1-compounds to formaldehyde. MMO is also able to oxidise methyl formate to formaldehyde and formate and ammonia to hydroxylamine (Colby et al., 1977; Dalton, 1977; Stirling and Dalton, 1980). The substrate specificity of the MMO in crude extracts of Methylosinus trichosporium is similar to that of Methylococcus capsulatus (Bath) whereas that of the system in Methylomonas methanica is more limited (Stirling et al., 1979), and in many respects is similar to the substrate specificity of particulate MMO of Methylococcus capsulatus (Bath) in which aromatic, alicyclic and heterocyclic compounds are not oxidised (Prior, 1985).

1.12 Energy Supply for the Production of Oxygenated Compounds

From an economical point of view, the use of whole cells rather than active cell extract preparations is clearly advantageous due to the

Table 1.11.1
Substrate specificity of MMO in vivo and in vitro

SUBSTRATE	PRODUCT	REFERENCE
Ethane	Ethanol	1, 2, 3, 4, 5
Propane	1-Propanol	2, 3, 4, 5, 6
	2-Propanol	1, 2, 3, 4, 5
Butane	1-Butanol	2, 3, 4, 5
	2-Butanol	1, 2, 3, 4, 5
Isobutane	Isobutanol	5
	tert-Butanol	5
Pentane	1-Pentanol	2, 5
	2-Pentanol	1, 2, 5
	3-Pentanol	9
Hexane	1-Hexanol	2, 9
	2-Hexanol	1, 2, 5
	3-Hexanol	9
Heptane	1-Heptanol	2, 5
	2-Heptanol	2, 5
Octane	1-Octanol	2, 5
	2-Octanol	2, 5
C_9-C_{16}-n-Alkane	C_9-C_{16}-n-1-Alkanol	8
Ethylene	Ethylene oxide	2, 4, 5, 6
Propylene	1,2-Propylene oxide	2, 4, 5, 6, 8
1-Butene	1,2-Butylene oxide	2, 4, 5, 6
trans-2-Butene	trans-2,3-Epoxybutane	2, 5, 6
	trans-But-2en-1-ol	2
cis-2-Butene	cis-2,3-Epoxybutane	2, 5, 6
	cis-But-2-en-1-ol	2
Butadiene	1.2-epoxybutene	4, 5
Isoprene	1.2-epoxyisoprene	5
Carbon monoxide	Carbon dioxide	9
Methanol	Formaldehyde	9
Chloromethane	Formaldehyde	6, 9, 10
Dichloromethane	(Carbon dioxide)	9, 10

Table 1.11.1 (continued)

SUBSTRATE	PRODUCT	REFERENCE
Trichloromethane	(Carbon dioxide)	9, 10
Bromomethane	Formaldehyde	5, 6, 9, 10
Fluoromethane	Formaldehyde	5
Dimethylether	Methanol	5, 6, 10
	Formaldehyde	5, 6, 10
Diethylether	Ethanol	6, 10
	Ethanal	6, 10
Methylformate	Formaldehyde	10
	Formate	10
Cyclopropane	Cyclopropanol	11
Methylcyclopropane	Cyclopropanol	11
Cyclohexane	Cyclohexanol	5, 10
Benzene	Phenol	5, 8, 10
	Hydroquinone	10
Toluene	para-Cresol	5, 8, 10
	Benzyl alcohol	8, 10
Ethylbenzene	ortho-Hydroxyethylbenzene	8
	para-Hydroxyethylbenzene	8, 11
	Phenylethanol	8, 11
Styrene	Styrene oxide	9, 11
	para-Hydroxystyrene	11
para-Methylstyrene	para-Hydroxymethylstyrene	11
Propylbenzene	para-Hydroxypropylbenzene	11
1-Phenylheptane	1-Hydroxy-1-phenylheptane	8
	1-Phenylheptane-7-ol	8
Substituted-anisoles	Substituted-phenols	12
meta-Cresol	(meta-Hydroxybenzaldehyde)	8
	(para-Hydroxybenzaldehyde)	8
para-Cresol	5-Methyl-1,3-benzenediol	8
meta-Chlorotoluene	Benzylalcohol	8
	meta-Hydroxybenzylalcohol	8
	para-Hydroxybenzylalcohol	8

Table 1.11.1 (continued)

SUBSTRATE	PRODUCT	REFERENCE
Pyridine	Pyridine-N-oxide	9, 10
Naphthalene	beta-Naphthol	11
	1,6-Naphthalenediol	11

( ) : further oxidised compound by dehydrogenases

1. Hou et al. (1981)
2. Dalton (1980)
3. Patel et al. (1980)
4. Hou et al. (1980)
5. Patel et al. (1982)
6. Stirling and Dalton (1979)
7. Patel et al. (1980)
8. Higgins et al. (1980)
9. Colby et al. (1977)
10. Dalton (1981)
11. Dalton et al. (1981)
12. Jerequel et al. (1984)

prohibitive cost of extracting the enzymes on a large scale, the need to regenerate the co-factor in cell free systems and the instability of enzymes, especially MMO. In this project, propylene oxide (PO) was selected as a target compound, for the biotransformation using methane-oxidising bacteria. In the production of PO, methanol is used as an electron donor because the cell has dehydrogenases present which can supply electrons to MMO. However, if extracted MMO is used as a biocatalyst, a cofactor regeneration system must be prepared. NADH can be used as an electron donor, but this compound is extremely expensive. Accordingly, it is clear that the whole cells are important for the production of PO. However, until the late 1970's cofactor regenerating systems were not paid attention to in the production system using methanotrophs. In early studies (Scott et al., 1981b; Dalton, 1980) an important factor in determining the rate and extent of whole cell biotransformations was thought to be the level of endogenous energy reserves. Scott et al., (1981b) reported that organisms harvested from carbon excess, nitrogen-limited cultures of Methylosinus trichosporium (OB3b) had a high level of poly-beta-hydroxybutyrate (PHB) and shows concomitantly faster propylene oxidation and greater product accumulation than those organisms with low levels of PHB from carbon-limited, nitrogen excess growth conditions. Similar observations concerning the energy supply can be found in another paper (Dalton, 1980).

The strong attention paid to the endogenous energy source may have been due to the poorly optimised whole cell assay systems used. At that time whole cell assays appeared to be performed under high biomass conditions. In high biomass, whole cell activity is not a true reflection of its actual activity since there may be limitations due to the level of oxygen, electron donor or substrate hydrocarbon (Stanley, personal communication). For example, the product propylene oxide (PO)

is sometimes produced in almost similar amounts with or without electron donor under high biomass conditions (Subramanian, 1986). In this case, specific PO productivity is relatively low and electron donation is not a limiting factor to PO production. If 50% of biomass is a poly-beta-hydroxybutyrate (PHB) and one mole of PHB can supply 2 moles of NADH, only 5.6 grams of PO could be produced from 10 grams of cells. This amount of PO is too small to make the process economically. Drozd (1986) noted that 10 grams of cell should produce 500 grams of PO during their life.

1.13 Is Methanol Dehydrogenase able to Supply Electons to the MMO?

In an attempt to discover the in vivo source of the electron donor for the MMO, Ferenci (1974) reported that carbon monoxide oxidation by whole cells of Methylomonas methanica was stimulated by ethanol, but that there was no NAD^+-linked alcohol dehydrogenase present in the cell extract (Ferenci et al., 1975). Stanley et al., (1983) also suggested that ethanol could act as an electron donor for the particulate MMO. Ferenci et al. (1975) proposed that ethanol could indirectly reduce NAD^+ by reversed electron transport. A different hypothesis was proposed by Prior (1985) who mentioned that NADH might not be the intermediate electron donor for the particulate MMO even though NADH is effective as a donor for the enzyme in vitro, and that reductant for the MMO from methanol and ethanol was generated via an electron transfer protein without the involvement of NADH. One possible candidate for this electron transfer protein might be a copper-containing protein that was only synthesized when cells were grown under conditions of copper excess and that this protein may have been capable of donating electrons directly to the particulate MMO without the involvement of NADH (Prior, 1985). However, these

investigations into the electron transport mechanism to MMO from methanol are still unresolved.

Many workers have demonstrated that ethanol could act as an electron donor to MMO, but these results obtained from their experiments were complicated by the fact that acetaldehyde, the product of ethanol oxidation, is further oxidised to acetic acid by formaldehyde dehydrogenase. This latter step could, in principle, give rise to reducing power for MMO since some formaldehyde dehydrogenases are an NAD^+-linked enzyme. Hazau et al., (1980) suggested that one could not readily differentiate between endogenously-derived electron donors and those arising from the oxidation of ethanol or acetaldehyde.

Leak and Dalton (1983) demonstrated that primary alcohols ranging C_2 to C_4 and their corresponding aldehydes were oxidised by obligate methanotrophs and reducing equivalents from each oxidation step could be utilised, in vivo, to stimulate MMO activity. They also reported that 5mM-acetate, propionate and butyrate also stimulated MMO activity apparently by stimulating the breakdown of PHB, subsequent metabolism of which gave rise to NADH. Methanol dehydrogenase probably provides electrons for MMO, but it is not conclusively resolved from the investigations by Leak and Dalton (1983). This subject will be discussed in Chapter 3.

1.14 The Stability of Biocatalyst

1.14.1 The Stability of the Soluble MMO

The stability of the biocatalyst is one of the most important factors for biotransformation systems. The stability of a multicomponent enzyme system such as the soluble MMO of Methylococcus capsulatus (Bath) (Woodland and Dalton, 1984a) is dependent on the

individual stabilities of the enzyme components. Each component may require different conditions or the addition of different stabilising agents to remain active. In practice, however, one of the components of the enzyme complex will be appreciably less stable than the others and therefore, in crude cell preparations will always appear to be, or will quickly become, the rate-limiting component of the enzyme complex.

Colby and Dalton (1976) reported, that the 25% of soluble MMO activity in cell extracts of Methylococcus capsulatus (Bath), was lost over 24 hours at 4°C. On the resolution of the soluble MMO from Methylococcus capsulatus (Bath) into its three components, DEAE-cellulose fractions A and B were found to be stable over a period of 24 hours at 0°C. The instability of the soluble MMO was due to a loss of 60 to 90% activity of the reductase component in DEAE-cellulose fraction C over a 20 hour period at 0°C (Colby and Dalton, 1978). All three fractions were stable when frozen in liquid nitrogen and stored at -80°C. A number of stabilising chemicals were tested for their effect on the stability of component C. Sodium thioglycollate (5mM) and dithiothreitol (5mM) were shown to stabilise as was NADH (5mM) with no loss of activity in 22 hours at 0°C. In a subsequent report by Woodland and Dalton (1984a), partially purified component A of soluble MMO from Methylococcus capsulatus (Bath) was shown to lose 40% of its activity over 72 hours at 4°C. A wide range of stabilising chemicals failed to prevent this loss of activity. Once purified, component A was not stable to freezing, but could be stored in 50% glycerol at 20°C for several weeks without loss of activity. This instability was attributed to the loss of iron from the protein, as activity could be restored by incubation with iron and dithiothreitol, though this effect was variable. Component B of the soluble MMO of Methylococcus capsulatus (Bath) was only stable after treatment with phenylmethylsulphonyl fluoride (PMSF), a serine protease inhibitor (Green and Dalton, 1985).

In the presence of 5mM sodium thioglycollate, crude preparations of component C were more unstable, losing 30% of their activity per hour. It was stable to freezing and could be stored at -70°C indefinitely without loss of activity (Lund, 1983). In crude cell extracts, made in either the presence or absence of 5mM sodium thioglycollate, component C was shown to be the limiting factor in soluble MMO activity (Pilkington, 1983).

In case of the soluble MMO of Methylosinus trichosporium (OB3b), crude cell extracts were unstable, losing all activity after 24 hours at 4°C (Stirling and Dalton, 1979a; Scott et al., 1981a). However, activity could be restored to these extracts by the addition of DEAE-cellulose fractions B and C from the soluble MMO of Methylococcus capsulatus (Bath). DEAE-cellulose fractions refer to proteins (A, B or C) eluted from the DEAE-cellulose column in the early stages of preparation of purified MMO. DEAE-cellulose fraction 1 (probably equivalent to protein A) of the soluble MMO of Methylosinus trichosporium (OB3b) lost all activity in 1 to 3 hours at 4°C and also when frozen in liquid nitrogen and stored at -80°C (Stirling and Dalton, 1979a). A number of stabilising chemicals, including PMSF, dithiothreitol and sodium thioglycollate had no effect on the stability of DEAE-fraction 1 (Stirling and Dalton, 1979a). On the other hand, Scott et al. (1981a) found that the soluble MMO of Methylosinus trichosporium (OB3b) was stable at liquid nitrogen temperature and noted that the stability of the soluble MMO in soluble extracts was enhanced by the addition of number of stabilising chemicals, particularly PMSF (1mM) and ditiothreitol (1mM). Storage of extracts under anaerobic conditions also protected the loss of soluble MMO activity.

Pilkington (1983) showed that 40% of the initial activity of soluble MMO from Methylosinus trichosporium (OB3b) was retained over 24 hours at 0°C. However, other chemicals showed variable stabilisations.

For example, other protease inhibitors such as aminophenylboronic acid and procans were used for their ability to stabilise the soluble MMO, but they were not as effective as PMSF.

1.14.2 The Stability of the Particulate MMO

There are few reports concerning the stability of particulate MMO. Prior (1985) suggested that the major difficulty with the preparations of particulate MMO was the instability of the activity of the membrane fractions. This loss of activity was found to be temperature sensitive. The loss of activity was 60-70% hr^{-1} at 45°C and 15-20% loss hr^{-1} at 0°C. Preparations were found to be stable at -20°C; this contrasts with the results of Tonge *et al.* (1979) who reported that the particulate MMO isolated from *Methylosinus trichosporium* (OB3b) was totally inactivated on freezing. The instability of the membrane-bound MMO from *Methylococcus capsulatus* is similar to the labile nature of membrane preparations from other organisms (Ferenci *et al.*, 1975; Ribbons, 1975; Colby *et al.*, 1975) and the fact that the preparation is unstable above 0°C was similar to the instability noted by Colby *et al.* (1975) for the soluble MMO from *Methylococcus capsulatus* (Bath).

During the preparation of soluble MMO from *Methylococcus capsultatus* (Bath), 1-10 mM of sodium thioglycollate is routinely added to the sample to act as a sulphydryl-group protecting reagent for protein C of the enzyme complex, but Prior (1985) reported that addition of this compound to particulate preparations led to reversible inhibition of MMO activity and did not provide any enzyme stabilisation. Furthermore, Prior (1985) found that the addition of dithiothreitol and glycerol, routinely used as a stabilising agent for cytochrome P-450, provided no effect. PMSF, the compound found to stabilise soluble MMO did not exhibit any increased stability, suggesting the loss of activity

was not due to degradation of the enzymes, but was due to the labile nature of the enzyme or proteins necessary for expression of full *in vitro* activity of the enzyme. The fact that the addition of sodium thioglycollate inhibited the particulate MMO reversibly may suggest that this compound may act as a metal-chelating agent, especially for copper ions, in a manner similar to thiourea as reported by Colby and Dalton (1976).

It has been demonstrated that purified soluble MMO and particulate MMO in cell extracts are obviously unstable and the extracted enzyme may not be used as a biocatalyst.

1.15 Inhibition and Inactivation of Biocatalyst

1.15.1 Inhibition of MMO by Chelators

Studies on methane-oxidising bacteria have shown that they are sensitive to a whole range of inhibitions, especially metal-binding agents (Hubley *et al*., 1975; Ribbons, 1975; Colby *et al*., 1975; Stirling and Dalton, 1977; Patel *et al*., 1976). These reports suggested that metal ions may be involved in methane oxidation. The problem with any such study is that in whole cells or crude membrane fractions the site of inhibition is not always clearly defined. Hence, the results of inhibition data obtained with heterogenous fractions should be treated carefully.

Stirling and Dalton (1977) demonstrated that the soluble MMO in cell extracts of *Methylococcus capsulatus* (Bath) was insensitive to the majority of inhibitors tested and was only inhibited by 8-hydroxyquinoline and the acetylenic compounds. Recently, Woodland and Dalton (1984a) have shown that soluble MMO contains two anti-ferromagnetically coupled iron atoms at its active site.

Accordingly, the lack of inhibition of this enzyme by metal chelating agent other than 8-hydroxyquinoline suggests that presumably these metal ions are shielded and not sensitive to the presence of metal ion chelators by virtue of their position within the protein molecule.

The particulate MMO of Methylococcus capsulatus (Bath) was found to exhibit a totally different inhibitor profile compared with soluble MMO. The particulate MMO was inhibited by metal chelators, thiol chelators and electron transport inhibitors (Prior and Dalton, 1985a). These properties closely resemble the pattern of inhibition described for the particulate system from Methylosinus trichosporium (OB3b) (Tonge et al., 1977), Methylomonas methanica (Colby et al., 1975) and Methylococcus capsulatus (Texas) (Ribbons, 1975; Stirling and Dalton, 1977), and suggests a close association of this form of the enzyme with metal ions and with membrane-bound electron transport proteins (Prior, 1985).

These inhibitors cannot be considered to be physiological since they do not occur during normal metabolism, whereas methanol and formaldehyde do satisfy this criterion. Another possible natural inhibitor could be nitrite which occurs as an intermediate of nitrate reduction or ammonia assimilation in methanotrophs.

1.15.2 Inhibition of Cell Growth by Nitrite

The mechanism of inhibition of bacteria by nitrite has not been well-explained. Rowe et al. (1979) reported that nitrite inhibited the active transport of oxygen uptake, and oxidative phosphorylation in Pseudomonas aeruginosa. They described that inhibition of respiration and active transport by nitrite occur co-ordinately. Furthermore, Yarbrough et al. (1980) showed that aldolases from Escherichia coli, Pseudomonas aeruginosa and Streptococcus faecalis as well as from rabbit muscle were inhibited by nitrite. It was also shown that nitrite

increased the proton conductance of the cytoplasmic membrane, resulting in a collapse of the proton gradient across the membrane.

Nitrite is an intermediate of nitrate reduction and also intermediate of ammonia oxidation, however, the function of nitrite in methylotrophs has not been well studied. Stanley (1977) reported that the growth of Methylococcus capsulatus (Bath) was inhibited when cells were inoculated into a medium containing a high (0.8 g/l) concentration of potassium nitrate. The reason for the lack of growth at the higher concentration of nitrate was presumed to be due to an effect of nitrite which accumulated within the culture. Stanley (1977) observed that the culture pH was lower in the flasks that did not grow and addition of potassium nitrite at the concentrations ranging from 0.5 g/l (7mM) to 2 g/l (28 mM) inhibited the cell growth.

1.16 Inactivation of MMO by Acetylene

Acetylene has been shown to act as a potent inhibitor of methane-oxidising activity in Methylomonas methanica (Colby et al., 1975); in Methylococcus capsulatus (Dalton and Whittenbury, 1976); and in Methylosinus trichosporium (Scott et al., 1981a). Acetylene is also a potent inhibitor in the oxidation of ammonia by the chemolithotrophic nitrifying-bacterium Nitrosomonas europaea (Hynes and Knowles, 1978). Cytochrome P-450 is inactivated during catalytic interaction with acetylenic compounds (White and Muller-Eberhard, 1977; White, 1978; Ortiz de Montellano and Kunze, 1980a). Past studies relating to acetylenic inactivation of cytchrome P-450 is reviewed by Walsh (1983) and Ortiz de Montellano (1986).

The first record of the inhibitory effect of acetylene on cell extracts of Methylococcus capsulatus (Bath) indicated that 3% acetylene in the assay mixture is sufficient to totally inhibit MMO activity

(Stirling and Dalton, 1977). In addition, it was reported that eight acetylenic compounds, tested for inhibition of MMO activity acetylene, propyne, but-1-yne and but-2-yne were found to be highly effective inhibitors of methane oxidation. However, it was also noted that inhibition-efficiency decreases not only with increasing chain length, but also by shifting the acetylenic bond away from the terminal carbon to a subterminal position. Acetylene has been shown to inhibit the MMO activity in cell extracts in other methylotrophs including Methylosinus trichosporium (Scott et al., 1981a), Methylococcus capsulatus (Texas) (Stirling and Dalton, 1977) and both soluble and particulate forms of MMO in Methylococcus capsulatus (Bath) (Stanley et al., 1983).

In early work on acetylenic compound-inhibition, Stirling and Dalton (1977) showed that when acetylene inhibited the MMO activity in the soluble fraction of cell extracts of Methylococcus capsulatus (Bath), the concentration of the acetylene in the assay diminished. They further demonstrated that the apparent loss of acetylene was dependent on the concentration of the extract present, and on the presence of NADH and oxygen. Attempts to identify a product for the apparent acetylene-oxidation were unsuccessful, because it was suggested that the inhibitor may have been bound to the enzyme. Recently, Prior and Dalton (1985b) have succeeded in showing that the [^{14}C]-labelled acetylene remained bound to the proteins, even after boiling-water treatment. This confirmed the theory that a product of acetylene-oxidation forms a strong bond with enzyme proteins. The soluble fraction of cell extracts of Methylococcus capsulatus (Bath), grown under conditions where soluble MMO activity was expressed, showed that the radio-labelled acetylene had bonded with a single polypeptide of molecular weight 54,000. This polypeptide corresponded to the alpha-subunit of protein A of soluble MMO (Woodland and Dalton, 1984a). Radio-labelled acetylene is also bound to a single polypeptide of

particulate MMO. However, in this instance, the polypeptide was found in the particulate fraction of cell extracts and had a molecular weight of 25,000. This corresponded to one of three proteins, which were induced at high copper:biomass ratios (Stanley et al., 1983; Prior and Dalton, 1985b). These were thought to be associated with particulate MMO-activity (Prior and Dalton, 1985a). From the above investigations it is clear that acetylene is an inactivator of MMO. Prior and Dalton (1985b) have proposed that acetylene is a suicide substrate of MMO and that a ketene is formed during mono-oxygenation of acetylene. Ketenes are notoriously reactive and inactivate by binding with neighbouring amino acids around the active site which may be involved in catalysis.

1.17 Inactivation of Biocatalyst by Epoxides

Epoxides are produced from alkenes by the action of MMO, however, epoxides are highly toxic for organisms. Ethylene oxide, which had been discovered at the middle of the 18 century, has been used since the 1930's as a sterilising agent. The effectiveness of propylene oxide (PO) as a gaseous sterilising agent was first reported by Mrak et al. in 1950. In 1958, FAD permitted the use of propylene oxide as a sterilising agent for some foods because of the suspicion that ethylene oxide may be a carcinogenic agent. Subsequently, many studies have been reported, notably by the Tamaratani-group (see Tamaratani, 1986). Table 1.17.1 shows the sterilising effect of PO on various micro-organisms reported by Tamaratani (1986). Gaseous PO is effective, but its activity as a sterilising agent is only half to a quarter of that of ethylene oxide (Bruch and Koesterer, 1961; Karaluk, 1971) due to the lower permeability of PO compared to ethylene oxide (Sykes, 1965).

The effect of PO on organisms is thought to be due to a direct alkylation of the biological molecules. Unlike many alkylating agents

Table 1.17.1

Sterilising effect of propylene oxide

for various organisms

(from Tamaratani, 1986)

Micro-organism	PO concentration (mg/l)	Inactivation time (min)
Xanthomonas phaseoli	0.3	45
Pseudomonas medicaginis	0.3	45
Ascochyta pisi	0.3	45
Fusarium solani	0.3	45
Fusarium cucurbitae	0.3	45
Verticillium sp.	0.5	45
Agrobacterium tumefaciens	0.5	60
Corynebacterium michiganense	0.5	60

Experiments were undertaken at 25°C.

the epoxides react with water without the liberation of acid and with anions or tertiary amines in aqueous media to liberate alkali. The characteristic biological action of the epoxides suggested that DNA may be their significant target *in vivo*. Several workers had noted the reaction of PO with guanosine in water (Lawley and Wallick, 1957; Brooks and Lawley, 1961; Haines *et al*., 1962) and Windmueller and Kaplan (1962) showed that ethylene oxide reacted with adenine nucleotides predominantly at the N-1 position.

However, the findings reported by Pochon and Michelson (1967) might be considered to suggest that PO was atypical, in that no reaction with DNA or polyguanosine could be detected in aqueous solution, whereas a reaction was reported at the N-1 position of thymidine and uridine. These apparent contradictions were finally resolved by Lawley and Jarman (1972) who showed that PO reacted with DNA in aqueous buffer solution at about neutral pH values to yield two principal products, identified as 7-(2-hydroxypropyl) guanine and 3-(2-hydroxypropyl) adenine. This reaction was investigated in 1.25M sodium acetate buffer at a PO concentration of 200 mM at 37°C for 7 days.

Tamaratani *et al*. (1980) suggested that PO induced single strand breaks of DNA *in vivo* in the studies on the mechanism of the disinfective action of propylene oxide toward bacterial spores. It was estimated that guanine was the primary target within DNA for reaction of PO. They concluded that the disinfecting action of PO towards the bacterial spore was alkylation of DNA. However, this study was also carried out at high PO concentrations (5% - 7% v/v) by Tamaratani (1986), who suggested that PO reversibly inhibited the germination enzymes, but did not inactivate them.

1.18 Inactivation of Biocatalyst by Radicals

In 1976, Hutchinson *et al*. proposed a possible role of free

radicals in the oxidation of methane by Methylococcus capsulatus (Bath). However, no evidence has been demonstrated on the formation of radicals during the oxidation of methane. Almost all aerobic organisms form the superoxide radical, $O_2^{\cdot -}$, and which is a major agent in the mechanisms of oxygen toxicity (Fridovich, 1978; Chance et al., 1979). However, the superoxide radical itself is fairly less reactive in aqueous solution (Sawyer and Gibian, 1979) and most of the damaging effects of superoxide radical-generating systems has been attributed to the superoxide radical-dependant formation of more reactive species, such as hydroperoxyl radical $HO_2^{\cdot}$ (Gebicki and Bielski, 1981), singlet O_2 (Khan, 1981) and especially the hydroxyl radical, $OH^{\cdot}$ (Halliwell, 1981) or species of equivalent reactivity (Cutteridge, 1982). The hydroxyl radical is highly reactive and is known to react rapidly with a wide variety of organic compounds in oxidative-generating systems in the presence of iron salts, apparently by the following mechanism.

$$Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

Net $$O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + OH^{\cdot} + OH^{-}$$

Rowley and Halliwell (1983) reported that copper ions at physiological concentrations can promote the formation of hydroxyl radicals or a species of equivalent reactivity. The reaction requires hydrogen peroxide and a reducing agent.

1.19 Stabilisation of Biocatalyst

There are several ways of overcoming inactivation which occurs when epoxides are produced from alkenes:

a) By stabilising the cells, using an immobilisation technique.

b) By modifying enzymes using chemical treatment or by using genetic engineering techniques.

c) By the addition of stabilising agents.

d) By the particular method for growing the cells.

e) By the selection of resistant mutants.

f) By the reactivation of inactivated cells.

Several scientists have succeeded in stabilising cell activity and prolonging the short life-span of biocatalyst. Chibata and Tosa (1984) immobilised Escherichia coli in polyacrylamide gel to produce aspartate from fumarate on a commercial scale. The half life of these immobilised cells was 120 days in this first application. The immobilising material was then changed to kappa-carrageenan, which was then treated with glutaraldehyde and polyethyleneimine (Sato et al., 1979). The half life was prolonged to 680 days as a result. Takata et al. (1983) also succeeded in stabilising the cell activity of Brevibacterium flavum for the commercial production of malic acid from fumaric acid by replacing acrylamide gel with kappa-carrageenan gel containing polyethyleneimine. The half life of catalyst was extended from 94 days to 243 days as a result of this particular study.

It is not clear why cell-life becomes stable under immobilised conditions. Takata et al. (1983) suggested, in their study of malic acid production, that the cation on the surface of the cells combines with kappa-carrageenan polyionically, and that this interaction makes the enzyme in the cells more stable.

CHAPTER 2

Materials and Methods

2.1 Organisms

Twenty two obligate methane-oxidising bacteria were used during these studies which were maintained as a stock culture in our laboratory. Methylococcus capsulatus (Bath) and Methylocystis parvus (OBBP) were used as typical organisms of Type I (also Type X) and Type II respectively throughout the study. Some organisms are not yet identified.

2.2 Media

A basic nitrate mineral salts medium (NMS: Whittenbury et al., 1970 b) which was modified by Stanley and Richards of this project, was mainly used throughout these studies for the routine growth of the organisms. The composition of this medium is given in Table 2.2.1. In order to prevent precipitation of the minerals in this medium, phosphate was added aseptically before use for solid medium or medium for batch liquid culture. When the medium was used for continuous culture phosphate was added together, the pH was decreased below 4 to avoid the precipitation using nitric acid. For solid medium, 15 g/l of Difco bacto-agar was added to the medium (minus phosphates) prior to sterilization. Sterile phosphate solution was added aseptically to the sterile mineral salts medium when the agar was cooling.

2.3 Maintenance and Growth

Cultures were maintained on basic NMS agar plates as described previously (Whittenbury et al. 1970b). The plates were incubated at 30°C for mesophilic methane-oxidising bacteria and at 42°C for thermotolerant species in plastic containers with methane from a gas

Table 2.2.1

Composition of Trace Element Solution used in Nitrate Mineral Salts (NMS) Medium

Compound	mg l^{-1}
$CuSO_4 \cdot 5H_2O$	500
$FeSO_4 \cdot 7H_2O$	500
$ZnSO_4 \cdot 7H_2O$	400
H_3BO_4	15
$CoCl_3 \cdot 6H_2O$	50
EDTA	250
$MnCl_2 \cdot 4H_2O$	20
$NiCl_2 \cdot 6H_2O$	10
$NaMoO_4 \cdot 2H_2O$	1000

N.B. This is the trace element solution for low copper medium. In order to produce high copper medium an additional 3mg/l should be added to the above list.

Nitrate Mineral Salts Medium

KNO_3	1g l^{-1}
$MgSO_4$	1g l^{-1}
$CaCl_2$	50mg l^{-1}
Fe/EDTA	3.6mg l^{-1}
Trace element soln.	1ml l^{-1}

Separate addition phosphate soln. (10%) 10 ml l^{-1}

10% Phosphate Solution

$Na_2HPO_4 \cdot 12H_2O$	644.4 g
KH_2PO_4	254.0 g
in 9 l of H_2O	Final pH = 6.8

bladder to give a concentration of about 50% (v/v) methane in air. Routine growth on liquid medium was achieved by using conical flasks containing 25 ml of sterilized NMS medium in 250 ml flask or medium sterilized using filter (Pleated capsule filter, Gelman Scientific Inc., Michigan, U.S.A.) in 2 l conical flasks which were inoculated with a small amount of methane-oxidising bacteria, sealed with a Suba-Seal cap (William Freemann and Co. Ltd., Barnsley, U.K.) in the case of 250 ml flask. Silicone plug was used for 2 l flask. After inoculation, methane was injected with 20% (v/v) methane in air as the carbon sources. The flasks were then incubated on a rotary shaker at 30°C or 45°C until the liquid becomes turbid. These liquid cultures were added to fermenter systems for large scale cultivation of the organism. Inoculum size was varied from 25 ml to 400 ml depending upon fermenter size.

Studies on the batch and continuous cultures of the organisms were performed in a variety of systems (MBR Bioreactor AG, Switzerland; New Brunswick Scientific, Edison, N.J., U.S.A.) with working volumes from 0.3 to 18 litres, using dilution rates from 0.002 to 0.25 hr^{-1}. Whole cells of Methylococcus capsulatus (Bath) and Methylocystis parvus (OBBP) for the preparation of experiment on stability, inactivation and reactivation experiment and the experiment on the IRP-reactor were mainly grown in a 10 l fermenter. Methane was used as a carbon source routinely.

Cultivation of Methylococcus capsulatus (Bath) in continuous culture with methanol as sole carbon and energy source was established by initially growing the culture on a methane/air gas mixture, then the MMO of the cells was inactivated by rapid addition of acetylene into the fermenter. Then methanol was added continuously at rates of 100-350 nmol/min/mg cells. Until this new cultivating method was found in this thesis, adaptation from methane grown culture to methanol culture was troublesome and took at least a few days to adapt cells on methanol

culture (Hou et al., 1979a; Prior, 1985). This method is convenient and rapid for studies which need a methanol grown cell.

2.4 Dry Weight Estimations of Cultures and Cell Suspensions

The concentration of biomass was normally determined by measuring the optical density of cell suspensions at 540nm in a spectrophotometer (Unicam sp. 500, PYE Unicam) using water as a standard reference. The relationship between optical density at 540nm and dry cell weight was estimated. Dry cell estimations were measured by the filtration of suitable volumes of culture through membrane filters (0.4$_{\mu}$m pore size, Oxoid Ltd., London, U.K.). The membranes then were dried out at 105°C.

2.5 Preparations of Cell Extracts

Whole cells were centrifuged at 10,000 x g for ten minutes once and resuspended in cold 20mM sodium/potassium phosphate buffer, pH7. Sodium thioglycollate (5mM) was added to the breakage buffer for preparations of the soluble MMO, this stabilized protein C of the enzyme (Colby and Dalton, 1978). Cells were broken by a single passage through a French pressure cell at 137MPa followed by centrifugation at 80,000 x g for one hour so yielding a soluble crude extract. The particulate MMO is inhibited by sodium thioglycollate (Stanley et al., 1983) and so preparations of this form of the enzyme were broken in the absence of thioglycollate.

2.6 Whole Cell MMO Activity (Wca)

Whole cell MMO assay was performed in 7ml conical flask

containing 0.9ml of cell suspension and sealed with rubber Suba-Seal cap. Three ml of propylene was added by injection through the Suba-Seal replacing the same volume of the gas phase of reaction flask. The flask was pre-incubated for 30 seconds in 90 oscillations per minute at 45°C,a Gyratory water bath shaker model G76 (New Brunswick Scientific Co. Ltd., Edison, N.J., U.S.A.) and then electron donor was added. The electron donor for MMO activity was normally 0.1ml of 10 mM methanol (final concentration of methanol was 1 mM). Usually final cell concentration in flask was adjusted at OD_{540} = 3. If necessary 0.1ml of 20mM of formaldehyde (final concentration was 2mM) or 0.1ml of 1M potassium formate (final concentration was 100mM) were used as the donors instead of methanol. After three minutes (including the period for injecting onto the gas chromatograph), a sample of the liquid phase (5 µl) removed from flask was injected to the gas chromatograph [2.1M x 4mm i.d. Porapak 2 (Waters Associates, Milford, Mass., U.S.A.)] with flame ionization detection for analysis of propylene oxide concentration.

2.7 Methanol Dehydrogenase Assay

Usually methanol dehydrogenase is assayed by the method described by Anthony and Zatman (1967), however this assay is for the cell extracts. Anthony (1982) described that the specific activity of methanol dehydrogenase in crude extracts from different bacteria varied between 4 to 1300 nmol/min/mg protein. This reflected to some extent the variety of growth conditions and methods of cell breakage and enzyme assay. On the study of inactivation and the reactivation of the inactivated cells, a more rapid and simple method was required with a small amount of sample.

As shown in the general introduction, methanol dehydrogenase has a wide range of substrate specificity, however formaldehyde dehydrogenase

has not. Using these two different properties of dehydrogenases, n-butanol oxidation rate was measured as a methanol dehydrogenase activity. Whole cell methanol dehydrogenase activity (methanol dehydrogenase activity) was performed with the same method of whole cell MMO activity, except the electron donor (methanol) was replaced by 30 mM n-butanol (final concentration 3mM). Butyraldehyde produced from butanol by methanol dehydrogenase was analysed by gas chromatograph. When propylene was not added to the flask, production rate of butylaldehyde was decreased.

2.8 Methane Monooxygenase Assay

Methane monooxygenase (MMO) activity in cell extracts was determined by gas chromatographic assay of propylene oxidation with NADH as an electron donor. The assay for the soluble enzyme was determined in a 7ml conical flask which contained 20mM sodium/potassium phosphate buffer, pH7 and sufficient soluble extract. Activity was measured with the same procedure of whole cell MMO assay except NADH was used as an electron donor at final concentration of 5mM in reaction mixture. Ethanol in the 2% NADH solution was extracted by diethyl ether then NADH was used as the electron donor. The particulate MMO fraction was assayed similarly.

2.9 Immobilized Cell-biofilm Reactor (IBF-reactor)

It has been considered that propylene oxide accumulated in an aqueous phase following the biotransformation of propylene, this accumulation inactivates the biocatalyst (Habest-Crutsen and de Bont, 1985; Subramanian, 1986). If PO can be separated from the reaction mixture, the biocatalyst should be stabilised. To determine the

critical concentrations of PO for the cells, an immobilized cell-biofils reactor was designed. Fig. 2.9.1 shows the design of equipment used to supply medium and control the temperature of the immobilized cells. The medium was saturated with a gas mixture of propylene and oxygen (usually 50-50%) in a small fermenter at least 30 minutes before the experiment started at a temperature of 45°C. Routinely 0.3mM methanol solution was added as an electron donor and if necessary PO was also added to this vessel. Medium was supplied to the filter via a peristaltic pump (Perista Pump, Atto Corporation, Tokyo, Japan) at a rate of 3.4 ml/min (flow rate was changed if necessary). Temperature was controlled at 45°C by a water bath connected to a thermocirculator (Churchill Instrument Co Ltd, Middlesex, U.K.).

The cells (routinely 1.0 mg dry weight) were deposited on one side of a membrane filter (0.2μm, 4.9 cm^2, Minisart NML, Sartorius GmbH, W. Germany) with a syringe. A needle inserted into a rubber plug was placed on the outflow side of the filter housing before removing the syringe to prevent free flow of media through the housing. The inlet side was attached carefully so as to avoid air bubbles entered into housing to the discharge of the pump. Then the outgoing side was connected to the down stream sampling line. The whole assembly was then placed into the water bath. After 2 minutes, medium was fed. The medium flow rate was determined by collecting the effluent from the filter. PO concentration was measured using gas chromatograph and PO productivity was calculated from the medium flow rate and biomass deposited on the filter.

2.10 Inactivation of Cells under the Conditions of PO Production and its Reactivation

The inactivation of cells under conditions of PO production and its

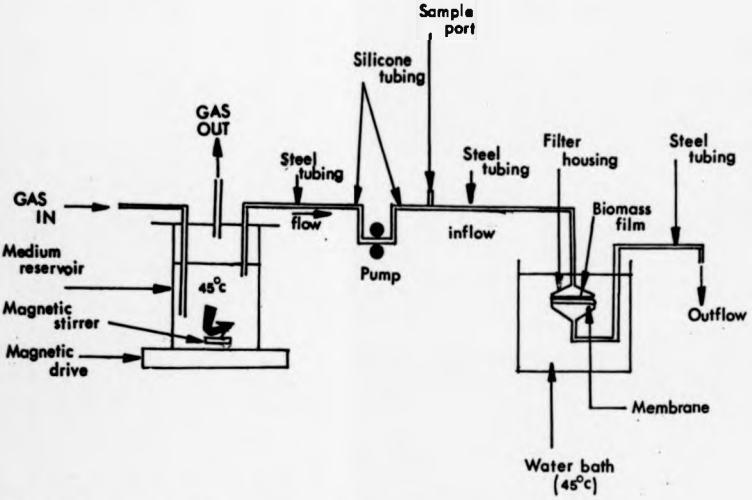


Fig. 2.9.1 The schematic diagram of the IBF-reactor

reactivation were undertaken using a 700ml fermenter. Normally, 500ml cells suspensions which contained particulate MMO were kept at 45°C when thermotolerant methanotrophs were used. When mesophilic methanotrophs were used, usually the temperature was controlled at 30°C except where specifically notified. The pH was controlled between pH 7.0 and 7.4 with 0.5N nitric acid. The agitation speed during the PO production stage was 1000 r.p.m. The reaction was started by charging 50% propylene in air and methanol. Methanol supply was varied for the aim of experiment, however usually 250 nmol methanol/min/mg cells were supplied. The reaction was ceased by cutting off both propylene and methanol supply. Then almost 20 VVM of air was supplied immediately in order to remove PO thoroughly from the reaction mixture. The amount of PO in the reaction mixture was monitored and when the PO concentration had decreased lower than 0.03mM, the supply of air was withdrawn and instead a mixture of methane and air was supplied as carbon and oxygen sources for reactivation. Normally 20% methane in air was used but when cells were highly inactivated, 50% methane in air was used because high DO inhibited the reactivation. The agitation speed was 400 r.p.m. during the initial reactivation period. Following the restoration of cell activity, the agitation speed was gradually increased to 1000 r.p.m.

2.11 Nitrite Determination

The concentration of nitrite was determined using the method demonstrated by Nicholas and Nason (1957). Test samples were centrifuged for 2 minutes in a Microcentrifuge (Quickfit Instrumentation, U.K.) to remove cells. To a test sample, made up to a volume of 0.2 ml, 0.1 ml of 1% sulfanilamide in 3M HCl was added followed by 0.1 ml of 0.02% N-(1-naphthyl) ethylenediamine hydrochloride

with shaking. As both the above solutions are light sensitive therefore they were kept in foil-covered bottle. The colour was left to develop at room temperature for 10 minutes and 0.2 ml of the sample was diluted with 3.8 ml of deionized water. Then the optical density was read at 540 nm. A standard curve was constructed by addition of 5 - 40 μmol nitrite to the assay. Standard solutions of nitrite were prepaired in dilute sodium hydroxide solution (25mg NaOH per 100ml) to prevent the liberation of nitrous oxide from the reaction of nitrite with carbon dioxide.

2.12 Protein Determination

Protein determinations were performed using the Bio-Rad protein assay system (Bio-Rad Laboratories Ltd., Watford, Herts., U.K.) with bovine serum albumin standards.

2.13 Gases

Methane chemically produced (technical grade) and propylene (pure grade) were obtained from both British Oxygen Company Ltd., London, U.K. and Electrochem Ltd., Stoke-On-Trent, U.K. Oxygen, nitrogen, hydrogen, argon, ethylene and acetylene were obtained from British Oxygen Company Ltd.

2.14 Chemicals

Compounds were obtained from the following manufacturers: BDH Chemicals, Poole, Dorset, U.K.; Aldrich Chemical Co. Ltd., Gillingham, Kent, U.K.; Wako Pure Chemical Industries Ltd., Osaka, Japan; Fluorochem Ltd., Derby, U.K.

CHAPTER 3

The Production of Oxygen containing Compounds by Methane-oxidising Bacteria

3.1 Reactions by Soluble MMO Cells

3.1.1 Introduction

The pioneering work of Colby et al. (1977) on the substrate specificity of MMO was the basis of the potential industrial exploitation of methanotrophs and their enzymes for biotransformation of hydrocarbons. Because the enzyme has a broad sustrate specify it may be possible to develop a biotransformation process for one substrate which could be used to produce oxygenated products from another substrates. Many companies, i.e. Exxon, ICI, Pfizer, and Dow have shown interest and developed biocoversions using methane-oxidising bacteria. However, no information has appeared to demonstrate development beyond laboratory scale experiments.

The most suitable products which can be produced by methanotrophs have been discussed by several workers (Hou, 1984a; Drozd, 1986; Subramanian, 1986; Large and Bamforth, 1988), and all of them have considered propylene oxide (PO) as the best product. However, no data have been shown on the comparison of PO and other compounds which can be produced by methanotrophs. At least 40 compounds have been reported as substrates of MMO so far, however the reactions and products which had been discovered were restricted to fairly simple substrates with the exception of pyridine.

In this Chapter consideration is given to the choice of a suitable substrate for further development in a biotransformation system. In particular those parameters which need to be evaluated before a final choice can be made are dealt with. It seems important to select a target compound from candidates for developing the production technology efficiently.

3.1.2 Experimental

The ability of whole cells of Methylococcus capsulatus (Bath) exhibiting soluble MMO activity, to oxidise a range of substrates was examined. The cells were harvested by centrifugation (12,000 x g) at 4°C, washed twice in ice-cold 20mM phosphate buffer, to give a concentration of 2mg dry weight/ 2ml. Four millilitres of gaseous substrates were added by displacement of air, liquid and solid substrates were added to give a final concentration of 2mM. The electron donor for all assays was 2mM methanol which was added to the system by injection after the cells and test substrates had been preincubated at 45°C on an orbital shaker for 30 seconds. Samples were removed after a known interval and products determined. Compounds were identified by comparing their retention times with those of authentic standards and quantitatively estimated by using Shimadzu GC-9A gas chromatograph.

3.1.3 Results and Discussion

Results are shown in Table 3.1.1. More than 30 new reactions and products were detected using cells containing soluble MMO. These can be classified into four categories as follows.

Category 1 - Para-hydroxylation of aromatic compounds -

The para-hydroxylation of aromatic compounds which were substituted by halogen, nitrile, amide or nitro were recognised. Meta-hydroxylation was also observed in nitrobenzene oxidation. Another non specific para-hydroxylation was observed in biphenyl oxidation.

Table 3.1.1

Oxidative products produced by whole cell of Methylococcus capsulatus (Bath) which contained soluble-MMO

Compound	Product
Category 1 -para-Hydroxylation-	
Methylbenzoate	para-Hydroxymethylbenzoate
Ethylbenzoate	para-Hydroxyethylbenzoate
Benzylchloride	para-Hydroxybenzylchloride
1,2-Dichlorobenzene	5-Hydroxy-1,2-dichlorobenzene
1,3-Dichlorobenzene	6-Hydroxy-1,3-dichlorobenzene
1,4-Dichlorobenzene	2-Hydroxy-1,4-dichlorobenzene
Fluorobenzene	para-Hydroxyfluorobenzene
Benzylnitrile	para-Hydroxybenzylnitrile
Phenylacetonitrile	para-Hydroxyphenylacetonitrile
Nitrobenzene	para-Hydroxynitrobenzene (50%) 3-Hydroxynitrobenzene (50%)
Phenylacetoamide	para-Hydroxyacetanilide
Diphenyl	4-Hydroxydiphenyl (97%) 2-Hydroxydiphenyl (3%)
Benzoate	not detected
Phenylsulphonate	not detected
Category 2 -Dehalogenation-	
Benzylchloride	Benzaldehyde
para-Chlorobenzylchloride	para-Chlorobenzaldehyde
Chloroethylbenzene	Phenylacetaldehyde
iso-Propylbenzylchloride	iso-Propylbenzaldehyde

Table 3.1.1 (continued)

Compound	Product
Category 3 -Expoxidation-	
Allylchloride	Epichlorohydrin
1-Chloro-2,3-butene	1-Chloro-2,3-epoxybutane
Methylacrylate	Epoxy methylpropionate
Methylcrotonate	Epoxy methylbutyrate
Allyl-n-butylether	Glycidyl-n-butylether
Allylbenzylether	Glycidylbenzylether
Allyl-iso-butylphenylether	Glycidyl-iso-butylphenylether
Diallylether	Allylglycidylether
Allylmethylester	Glycidylmethylester
Allyl-iso-butenoate	Glycidyl-iso-butenoate
Allyl-2-ethyl-hexylether	not detected
1-Phenyl, 1-methyl-ethylacrilate	not detected
per-Fluoropropylene	not detected
Category 4 -Miscellaneous (alpha-hydroxylation)-	
Ethylacetate	unknown product
Methylethylketone	1-hydroxy-2-ketobutane

The 4-hydroxy(para position) biphenyl was a major product (97%), however, less than 3% of 2-hydroxy(ortho) biphenyl was detected. Di-substituted phenyl-compounds were also oxidized and gave one product from each substrate, however no general rule governing the oxidation of di-substituted compounds could be found. Almost all the substrates tested were oxidised and gave product(s), but acidic compounds such as benzoate or phenylsulfonate were not oxidised by soluble-MMO cells.

Category 2 - Dehalogenation -

The end product of oxidation of halogenated C_1-compounds by MMO is formaldehyde. However, dehalogenation of C-C bond compounds has not been reported. Halogenated compounds which are directly substituted on the benzene ring were not dehalogenated, but produced a mono-hydroxy-halogenated compound as mentioned previously. However, aromatic compounds which have a halogenated alkyl group were dehalogenated and produced a corresponding aromatic aldehyde. No hydroxy-haloalkylphenyl compound was detected.

Category 3 - Epoxidation -

Many new epoxidation reactions were detected by soluble-MMO cells. Allylchloride was epoxidized to epichlorohydrin but acrolein, which might be a dehalogenated product of allylchloride, was not detected. Allyl ether, allyl ester were also oxidised and the corresponding epoxides were formed.

Unfortunately glycidyl ether, glycidyl ester were decomposed gradually during the course of the reaction. Perfluoropropylene was not oxidised.

Category 4 - Miscellaneous -

Methylethylketone was oxidised to 1-hydroxy-2-keto-butane and 3-hydroxy-2-keto-butane but 4-hydroxy-2-keto-butane was not formed.

From previous reports presented by many workers and data presented in this thesis, more than a hundred compounds can be produced by MMO. This substrate specificity is broader than the monooxygenase of Nocardia (Furuhashi, 1986), Corynebacterium (Ohta and Tetsukawa, 1978; Ohta et al., 1985) or Nitrosomonas (Drozd, 1980; Hyman and Wood, 1984), especially for the oxidation of aromatic compounds. Perhaps the most interesting biotransformation catalyzed by methanotrophs are concerned with the production of epoxides, because these compounds include several important bulk chemicals and many fine chemicals. However, alkene-oxidising bacteria such as Nocardia and Corynebacterium produce optically active epoxides. Unfortunately, the soluble MMO of Methylococcus capsulatus (Bath) gave a racemic mixture of epoxides (Waters, 1982). Subramanian (1986) also demonstrated that epoxybutane produced by Methylococcus capsulatus and Methylosinus trichosporium was a racemic mixture of R and S. Murakami (personal communication) observed that styrene oxide produced by soluble-MMO cells was racemic and Stanley and Suzuki (unpublished data) also obtained results in this project that propylene oxide produced by particulate-MMO cells of Methylococcus capsulatus (Bath) and Methylocystis parvus (OBBP) were both equal mixtures of optical isomers. Recently, Ono et al. (1988) have demonstrated that Methylosinus trichosporium (OB3b) produces a slight excess of the R-form of propylene oxide (R:S = 57:43) and S-form excess of butylene oxide (R:S = 36:64). Although highly enantioselective oxidation is clearly not a possibility, however, regio-selective oxidations were observed. These are summarised below:-

a) Compounds in which at least three carbon atoms are arranged around a central carbon atom and providing the fourth group is not a hydrogen atom are not oxidised by MMO.

b) When compounds have different substituents in their molecular structure, MMO oxidises the moieties with an order of preference as shown below: olefin epoxidation: degradation of ether or ester : hydroxylation of cyclic carbon if halogenated alkyl group is not present: dehalogenation of halogenated alkyl group if cyclic carbon is not present: hydroxylation of cyclic carbon: hydroxylation of alkyl associated with cyclic carbon: hydroxylation of methylketone.

c) When a compound is substituted by an acidic moiety, the compound is not oxidised by MMO. However, if the carboxylic acid is esterified then oxidation can occur. Higgins et al (1981a) have reported that alkylbenzenes are oxidised to para-hydroxy alkylbenzenes and phenylcarboxylic acids but they did not observe any hydroxy-phenylcarboxylic acid formation. These results support above theory.

d) Methylketones are further oxidised by MMO with the formation of hydroxyketones.

e) In nitrobenzene oxidation, unusual meta-hydroxylation occurs. This may be due to the electron-donating property of the nitro-moiety.

The products from previous reports presented by many workers and presented in this thesis can be classified into a few groups from the reaction patterns. In the next Section, reactions and compounds are

evaluated from the economical point of view and screened to select the target.

3.2 The Evaluation of Reactions and Compounds

3.2.1 Introduction

There are several parameters to consider when a particular biotransformation by methanotrophs is evaluated as a possible commercial process. These are summarised in Table 3.2.1. The best way to evaluate each compound is to compare the production cost and market size, however it is usually very difficult to estimate the production cost without any experimental data. However, one method that can be used is to roughly estimate the economics of production by comparing the material price (substrate) and product price. By comparing the price difference and market size, it becomes possible to judge which compounds may be economically viable to produce this way. Material price and market price must be a price which is accepted for the industrial trade and not the price sold as reagent for laboratory use. Market size also has to be carefully evaluated if market size is small the percentage of manpower costs in the production cost increases and this cost must be added on the production cost. In this Section, all the compounds which have been reported by others and also in this thesis were evaluated to select a target compound which should be studied further in this project. The production schemes are summarized in Table 3.2.2.

3.2.2 The Production of Primary Alcohols

Direct-oxidation of alkanes is very difficult by chemical method, however, microorganisms can readily perform this reaction.

Table 3.2.1

Parameters for evaluating the possible commercial exploitation of biotransformation procedure

Parameters	Item
Biological	(1) Yield and Selectivity - degradation of product
	(2) Productivity - may sometimes be possible to increase by the optimization of cultivation or reaction conditions.
	(3) Toxicity of Substrate or Product - concentration of product often strongly affects activity of biocatalyst, substrate toxicity is less important because it may be possible to control that by careful control of the feeding method.
Engineering	Recovery of Product - evaporation or crystallization from reaction mixture is best, extraction, absorption or a combination of absorption and extraction is less economical.
Market	Future Market Size - the future market when process will be completed is important, market stability, development of new competitive technology, production cost.

Table 3.2.2

Grouping of the Reactions by MMO

(1) Production of Primary Alcohols

Substrates		Products
$R-(CH_2)n-CH_3$	$\xrightarrow[MMO]{}$	$R-(CH_2)n-CH_2OH$

n : 0 - 9 ; R : H, CH_3

(2) Production of Secondary Alcohols and Methylketones

$$R-(CH_2)n-CH_2-CH_3 \xrightarrow[MMO]{} R-(CH_2)n-\underset{OH}{CH}-CH_3$$

$$R-(CH_2)n-\underset{OH}{CH}-CH_3 \xrightarrow[\text{Chemically}]{MDH\ or} R-(CH_2)n-\underset{O}{C}-CH_3$$

n : 1 - 8 ; R : H, CH_3

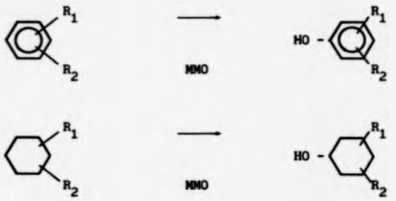
(3) Production of Epoxides

$$R_1-(CH_2)m-CH=CH-(CH_2)n-R_2 \rightarrow R_1-(CH_2)n-\underset{O}{C}-C-(CH_2)n-R_2$$

m+n : 0 - 8 (linear or cyclic); R_1, R_2: H, alkyl, halogen, ether, ester, phenyl, cycloalkyl, alcohol

Table 3.2.2 (continued/...)

(4) Production of Hydroxy-cyclic-compounds



R_1, R_2 : H, alkyl, phenyl, halogen, nitro, nitril, amide

(5) Production of aldehyds from halogenated compounds

$R - (CH_2)n - CH_2Cl \longrightarrow R - (CH_2)n - CHO$

R : phenyl n : 0 - 3

Unfortunately, substrate specificity of methanol dehydrogenase is very wide (see General Introduction) and primary alcohols produced by MMO are further oxidised to the corresponding aldehyde by the methanol dehydrogenase. If methanol dehydrogenase-minus mutants are obtained and this organism can grow on methanol using non-specific formaldehyde dehydrogenase, accumulation of primary alcohols may be possible. Temperature sensitive methanol dehydrogenase mutants grow at the permissive temperature and bioconvert at the non-permissive temperature. However, isolation techniques for mutants in methanotrophs is still difficult and development of this area would be necessary (Higgins et al., 1981; Haber et al., 1983).

In case of primary alcohol production, selectivity for primary alcohol is low (Colby et al., 1977; Stirling et al., 1979) and more than 50% of the product may be a secondary alcohol, even if primary alcohol accumulation could be possible.

3.2.3 The Production of Secondary Alcohols and Methylketones

As mentioned before, the selectivity for secondary alcohols is not high from the results of substrate specificity obtained with cell extracts of MMO. Moreover some organisms can oxidise further these secondary alcohols to methylketones by the secondary alcohol dehydrogenase (Bellion and Wu, 1978; Wolf and Hanson, 1978; Hou et al., 1979c). If activity of secondary alcohol dehydrogenase could be enhanced, and the selectivity of secondary alcohol is high, the production of methylketones, especially methylethylketone from butane is attractive. In the biological process, co-factor may be recycled from the secondary alcohol oxidation automatically. The merit of using a secondary alcohol as an electron donor will be discussed later in this Chapter.

3.2.4 The Production of Epoxides

The production of epoxides can be classified into three types which are short chain epoxides (C_2 - C_4), long chain expoxides > C_4 and glycidyl ethers or esters.

(1) The production of short chain epoxides

The selectivity of short chain epoxide from olefin is approximately 100% and productivity is also high. Some of them have a big market. Each of them is further characterised later in this Section.

(2) The Production of long chain epoxides

Nippon Mining Company (Furuhashi, 1986) has been successful in the commercialization of these compounds recently using alkene-oxidizing bacterium, Nocardia corallina B276. Their products are highly enantiomeric (R). To compete with this technology, using methane-oxidising bacteria appears to be difficult.

(3) Glycidyl ethers and glycidyl esters

Many of these compounds are useful intermediates for the production of beta-blockers (see Furuhashi, 1986). However as mentioned before, glycidyl ethers and glycidyl esters are decomposed by methane-oxidising bacteria, probably by the action of MMO.

3.2.5 The Production of Hydroxy-cyclic-compounds

The hydroxylation of cyclic-compounds is highly selective on para

position except ethylbenzene, meta-cresol, meta-chlorotoluene, nitrobenzene, biphenyl and naphthalene. When one of the ring carbons is hydroxylated, ring carbons are no more oxidised except phenol, para-cresol and 1-naphthol. By chemical means direct oxidation of cyclic-compounds to the corresponding hydroxy-cyclic compounds has not been commercially successful except cyclohexanol. Substituted phenols such as para-hydroxyacetanilide are attractive for targets. Detail of these compounds are discussed later.

3.2.6 The Production of Aldehydes from Halogenated Compounds and Others

The oxidation of 1-chlorotoluene and 2-chlorethylbenzene by cells containing soluble MMO produced benzaldehyde and phenylacetaldehyde respectively. However these compounds are difficult to accumulate by biotransformation using methanotrophs because these are further oxidised by either chemical and/or biological routes in water. Production of benzoic acid and phenylacetic acid from toluene and phenylethylbenzene respectively (Higgins et al., 1980; Patel et al., 1980; Dalton, 1980, 1981) indicate that aromatic aldehydes are oxidised by methanotrophs. Substrates are different in the dehalogenation and in the hydroxylation process but it can be obtained, the same products using same organism reveals the diversity of methanotrophs on biotransformation.

3.2.7 Comment

The results on the evaluation of compounds which have been discussed in this Chaper are summarised in Table 3.2.3. Short chain

Table 3.2.3

Evaluation of products by MMO

	Yield[1]	Selectivity[2]	Productivity[3]	Market[4]	Recovery[5]
Alcohols					
Primary alcohols	□	□	△	○	□
Secondary alcohols	△	□	△	○	□
Hydroxy cyclic compounds	○	○-△	△-○	△	□
Aldehydes and Ketones	△	□	□	○	△
Epoxides					
Short chain epoxide	○	○	○	○	△
Long chain epoxide	○	○	△-□	△-□	□
Cyclic epoxide	○	○	○	△-□	□
Glycidyl ethers and esters	□	○	△-□	○-△	□

○ good

△ moderate

□ not good

1) Degradation or stability of product

2) Single or by-product

3) Specific activity for each substrates

4) Market size

5) Ease of separation of product

epoxides and hydroxy-cyclic-compounds obtained comprehensibly high scores.

3.3 Selection of Target Compound and Discussion

The more detailed information on short chain epoxides and hydroxy-cyclic-compounds is listed in Table 3.3.1. Several compounds emerge as being potentially suitable candidates for further study. From the Table, it is clear the propylene oxide and epichlorohydrin in the group of short chain epoxides and substituted phenolic compounds in the group of hydroxy-cyclic-compounds are suitable as the targets for biotransformation without taking into account product toxicity and recovery difficulties.

Propylene oxide and epichlorohydrin are extremely valuable products because of their ability to undergo a variety of reactions. PO is produced industrially by either the chlorohydrin or the oxirane process. Both of these processes require multiple steps and depend heavily on the price of chlorine in chlorohydrin process and market price of by-product, such as styrene or t-butanol in the oxirane process. Many chemical direct oxidation processes have been investigated, but are still not beyond the laboratory scale (Table 3.3.2).

Direct oxidation is the most desirable route for PO and epichlorohydrin production. Many companies have been interested in PO production by microorganisms, but these biological routes are only at the laboratory scale (see Chapter 10) The reasons for their lack of development are:-

a) low productivity

b) high energy requirement

c) low stability of biocatalyst.

Table 3.3.1

Economic comparison of Products

Compounds	Possibility	Conclusion
Short chain epoxides		
Ethylene oxide	□	Direct oxidation by chemical process
Propylene oxide	○	
Butylene oxide	△	Market is not big (2,000 - 5,000 T/Y world)
Epichlorohydrin	△	Recovery
Glycidol	□	Decomposition of product
Hydroxy cyclic compounds		
Phenol	□	Chemical process is cheap
Cyclohexanol	□	Direct oxidation by chemical process
Naphthol	□	Low selectivity and low productivity
Chloro phenol	△	Market is not big
Cyano phenol	△	Market is not big
P-hydroxy acetanilide	△	Market is not big
Nitro phenol	△	Market is not big Selectivity is low (can separate)
Hydroxy biphenyl	△	Market is not big

○ high △ medium □ low

Table 3.3.2

Direct epoxidation processes of propylene by chemical method (laboratory scale)

Process	Company	Reference (Patent No.)
A. Vapour phase oxidation process	ICI	US 43490738
	Union Carbide	CA 986127
B. Liquid phase oxidation process	Air Products & Chemicals	US 4256649
	Texaco	US 4,420,625
	B.P.	GB 1,582,261
	Celanese	US 4,383,904
C. Cooxidation with an aldehyde	Halcon	GB 2008113
	Union Carbide	US 1,012,155
D. Method using oxidizing agent regenerated by molecular oxygen	Allied Corp.	US 4,356,311
	Dow Chemical	US 4,120,877
	Halcon	US 4,290,959

Another parameter for the evaluation of these compounds is the recovery method from the reaction mixture. PO can be separated by gas scrubbing.

Consequently, the author selected PO production as the most likely biotransformation to succeed in an industrial process. However if a more effective separation method for epichlorohydrin, butylene oxide or substituted phenolic compounds can be found from reaction mixture, then these compounds may be attractive targets for biotransformation by methanotrophs.

3.4 The Energy Supply for the Production of PO

3.4.1 Introduction

The nature of the MMO reaction is such that a supply of NADH is necessary to maintain activity. Therefore an electron donor is necessary to produce PO by whole cells of methane-oxidising bacteria. The efficiency of energy supply and the type of electron donor used will strongly affect the PO production cost. It is well known that methanol, formaldehyde and formate can be used to supply electrons for stimulated PO production. Methanol is cheaper than both formaldehyde and formate and has the potential to produce more electrons from its oxidation. However, it has not been proved that electrons can be supplied directly to MMO through the oxidation of methanol to formaldehyde by methanol dehydrogenase. To find out whether methanol dehydrogenase is capable of supplying electrons to the MMO would provide important information on this area of metabolism.

Moreover, it would provide useful knowledge on the theoretical maximum possible yield of PO from this electron donor. As mentioned in

the General Introduction, the substrate specificity of methanol dehydrogenases are extremely wide, but only C_1 to C_4 linear primary alcohols (Leak and Dalton, 1983) and short chain secondary alcohols are known to be capable of supplying electrons. Usually substrate specificity of whole cells are more restricted than extracted enzymes. However, it appeared that a wider range of primary alcohols might supply electrons to the MMO. Other electron donors such as methane and hydrogen are also discussed in this Section.

In this thesis, two methanotrophs: Methylococcus capsulatus (Bath) and Methylocystis parvus (OBBP) were mainly used for experiments. Methylococcus capsulatus (Bath) is the most well studied strain of methanotroph, so it appeared to be the most suitable for study. Furthermore, this organism grows well and produces PO at 45°C. This property is very important for the development of a PO production process. Methylococcus capsulatus (Bath) is a Type I (also Type X) methanotroph. To compare results of the important experiments a Type II methanotroph, Methylocystis parvus (OBBP) was selected because this organism also grows well and its growth temperature is relatively high (37°C) for the Type II methanotrophs.

3.4.2 Alcohols as Electron Donors

The effect of alcohols as electron donors was investigated using the same method of $\dot{V}_{CH_4}$ measurement as shown in Materials and Methods. The effect of alcohols on the production of PO are shown in Table 3.4.1. Surprisingly, many of the alcohols tested including diols and aromatic alcohols acted as electron donors. Especially in Methylocystis parvus (OBBP) where many primary alcohols could be used as electron donors.

Throughout this experiment, several features become clear.

Table 3.4.1

The Effect of Alcohols as Electron Donors

Strain	Electron donor	Concentration(mM)	PO productivity (nmol/min/OD=6)
M. parvus (OBBP)	- (control)	-	50
	methanol	1	320
	ethanol	2	244
	ethylene glycol	50	255
		100	288
	methoxy ethanol	3	189
	ethoxy ethanol	3	168
	n-propanol	3	295
	1.3-propylene glycol	50	189
	1-chloro-3-propanol	3	192
	n-butanol	3	160
	crotyl alcohol	3	224
	1.4-butanediol	50	204
	n-hexanol	1.5	197
	phenylethyl alcohol	3	238
	i-propanol	3	48
	1,2-propylene glycol	3	44
		100	31
	2-chloro-1-propanol	3	40
	propion aldehyde	3	52
	acrolein	3	24
	2-metyl-1-propanol	3	43
	n-butyraldehyde	2	48
	crotonaldehyde	1.5	45
	Benzyl alcohol	3	36
	n-octanol	saturated	45
M. capsulatus (Bath)	-	-	20
	methanol	1	314
	ethanol	3	94
	ethylene glycol	100	92
	n-propanol	3	194
	allyl alcohol	3	184
	n-butanol	3	122
	1.4-butanediol	50	28

PO productivity was measured at 45°C.

a) For primary alcohols, methanol was the most effective electron donor, however C_3 - C_6 alcohols could supply electrons at more than 50% of the methanol rate in Methylocystis parvus (OBBP).

b) No clear evidence was obtained that propionaldehyde or butyraldehyde could supply electrons to MMO. This suggested that all of the energy supplied by the oxidation of 1-propanol or 1-butanol were supplied directly through the methanol dehydrogenase to the particulate MMO in Methylocystis paruvus (OBBP) and Methylococcus capsulatus (Bath).

In 1983, Leak and Dalton reported that C_3 - C_4 aldehydes provided energy for MMO in Methylocystis parvus (OBBP). They used OBBP strain at high biomass (OD_{540} = 20) with a specific activity of 11 nmol/min/OD=4 when butyraldehyde was used as an electron donor. However, in this thesis low biomass (OD_{540} = 3) was used and even endogenous specific activity was far higher (40 nmol/min/OD=4) than the activity they obtained. Stimulation by the addition of butyraldehyde might be masked by high endogenous activity in this experiment.

c) Alpha-omega-diols also supplied energy for the particulate MMO when their concentration in the reaction mixture was high. No effect was observed when 3mM ethylene glycol was used as a donor, but 50 and 100mM of ethylene glycol supplied energy. It seems that low permeability of glycols may be responsible for this phenomenon.

d) When the beta-carbon is substituted with hydroxyl, halogen or carbon, the alpha-alcohols were not oxidised by methanol dehydrogenase. Therefore it appears that the active site of the

methanol dehydrogenase seems to be a narrow crevice and its diameter is less than the C-C bond distance. Moreover, the active centre is probably located at about the C-C bond length from the entrance. This assumption is based on observations concerning aromatic alcohol oxidation. Benzylalcohol did not supply energy for MMO (no stimulation of PO production) but phenylethylalcohol (beta-phenethyl alcohol) was effective.

Patel et al. (1980) had advocated that aromatic alcohols were not substrates of methanol dehydrogenase. Their conclusions were derived from the result on oxidation of benzylalcohol. However, if they had carefully considered their own result it appear to be not difficult to predict the oxidation of phenylethylalcohol by methanol dehydrogenase. The active site of methanol dehydrogenase is illustrated in Fig 3.4.1.

3.4.3 Methane as an Electron Donor

Theoretical studies suggested that organisms growing on methane would be limited by NADH supply unless methanol dehydrogenase supplied reducing power to MMO (van Dijken and Harder, 1975; Anthony, 1978). From the results shown in the last Section, it becomes more obvious that methanol dehydrogenase can supply electrons to the particulate MMO. This indicates that if methane is oxidised to carbon dioxide completely at least one mole of PQQH could be available for the oxidation of other compounds since NAD^+-linked formaldehyde and formate dehydrogenase activities have been demonstrated in methanotrophs (Patel and Hoare, 1971; Stirling and Dalton, 1978).

If methane works as an electron donor for the production of PO, it is as attractive as methanol. There is a small amount of evidence to

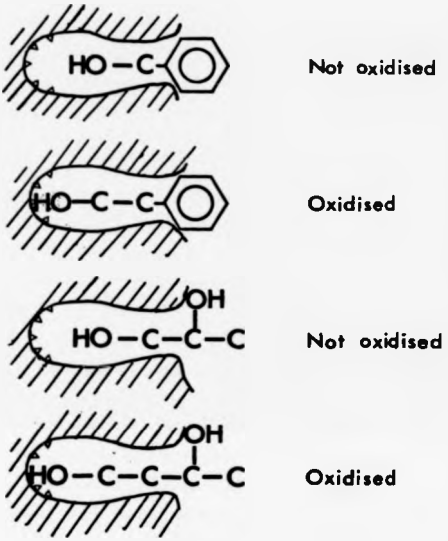


Fig. 3.4.1 The active site of methanol dehydrogenase

suggest that methane per se can act as an electron donor in the whole cell oxidation systems of methanotrophs. O'Neill and Wilkinson (1977) reported that low levels of methane stimulated nitrite production from ammonia, whereas high levels were inhibitory. This phenomenon could probably be explained by assuming that low levels of methane acted as an electron donor for ammonia oxidation, whereas high levels of methane competed with ammonia at the active site of MMO, so that ammonia oxidation was inhibited. Another example was that of Higgins et al. (1979) who used methane in the reaction mixture in their study on the substrate specificity of Methylosinus trichosporium (OB3b). No explanation of why methane was added to the reaction mixture was provided. Although, this indicates that methane can act as an electron donor.

Table 3.4.2 shows evidence that methane acts as an electron donor for PO production in Methylococcus capsulatus (Bath). When a gas mixture of methane, propylene and air was supplied to the reaction mixture, PO productivity was significantly increased compared with the control in the absence of methane. Methane and propylene are both substrates of MMO and these substrates compete with each other. However, the difference in the case of ammonia oxidation is that MMO has a high affinity for propylene. In the methane and propylene mixture atmosphere, methane and propylene may be oxidised alternatively by MMO and then methanol is further oxidised to supply electrons to MMO.

3.4.4 Hydrogen as an Electron Donor

Another economical electron donor for the production of PO is hydrogen. Some methanotrophs produce hydrogen as an electron donor by the action of hydrogenase (Kawasura et al., 1983). In the study of nitrogenase activity, Dalton and Whittenbury (1976) reported that

Table 3.4.2

Effect of methane as the electron donor on Wca

Gas composition (v/v) Propylene : Methane : Air			Wca (nmol/min/mg cells)
0.2	1.8	5	21
0.4	1.6	5	45
0.6	1.4	5	79
0.8	1.2	5	108
1.0	1.0	5	140
1.2	0.8	5	106
1.4	0.6	5	87
1.8	0.2	5	40
2.0	0	5	29
Control: 1.0 mM methanol as the electron donor (Propylene : Air = 2 : 5)			474

hydrogen could be metabolized by Methylococcus capsulatus (Bath) to provide co-factors for nitrogenases. Stirling et al. (1977) suggested that hydrogen could be used for the production of oxygenated compounds by methanotrophs, but no data was given. Stanley (personal communication) found that hydrogen was effective as an electron donor for the production of PO in Methylococcus capsulatus (Bath), however the efficiency of hydrogen as an electron donor was dependent on the culture conditions since hydrogenase was sensitive to oxygen. He suggested that the cell had to be cultivated under oxygen-limited conditions if hydrogen was to be effective as a donor in this study. Furthermore no stimulation was observed when hydrogen was used as an electron donor for Methylocystis parvus (OBBP) which was cultivated under oxygen-limited conditions which may suggest that this organism does not possess hydrogenase activity.

3.4.5 Discussion

Electron donors described in this Chapter can be classified into two groups:- Group A: C_1-compounds and hydrogen from which water and/or carbon dioxide are by-products; Group B: compounds which contain carbon-carbon bonds from which by-products are produced. From Group A, methanol, hydrogen and methane are better electron donors than formaldehyde or formic acid from an economical point of view. Formaldehyde is more toxic than the other electron donors and more expensive than methanol.

Formic acid is also more expensive and is less efficient as an electronic donor than methanol. Moreover methanotrophs require relatively high concentration of formate for the maximum productivity (50 - 150 mM). Hydrogen is the cheapest compound per unit mole of NADH

production. However hydrogen is highly explosive and therefore dangerous to use.

Group B compounds: 2-propanol and 2-butanol are suitable compounds if the secondary alcohol dehydrogenase activity is high enough to supply electrons to the MMO. Furthermore the by-products arising from the secondary alcohols have large markets and by-product credit may be possible if the resultant methylketones can be recovered economically from the reaction mixture. Practically, secondary alcohol dehydrogenase activities which have been reported by Hou et al. (1979d) are low. Therefore isolation of new strains or selection of mutants with elevated secondary alcohol dehydrogenase activities will be required before these compounds could be considered as possible economically viable electron donors.

Consideration of economical and biological view points indicate that methanol and methane be selected for further study, however, hydrogen should be studied when PO production technology is advanced. Best and Higgins (1983) have advocated that formate is preferred to methanol, because although providing more reducing power, methanol inhibits MMO, by acting as an alternative substrate. However, the inhibition by methanol can be avoided by controlling the methanol concentration in the reaction mixture by careful addition to the biotransformation. Hence, methanol appears to be more advantageous from the point of energy efficiency and price.

3.5 The Cultivation of High Activity MMO Cells

3.5.1 Introduction

In the previous Sections a target compound and suitable electron donors for a possible bioconversion process have been selected. The

other parameter which should be investigated before studying PO production is cell activity, especially whole cell MMO activity (Wca). Drozd (1986) suggests that for high volume, low cost products, it is important to maximise productivity as this influences capital cost. The reasons why a PO production process using methanotroph has not been developed far beyond the laboratory scale is low productivity and instability of the system.

Many investigations have demonstrated the activity of MMO in both cell extracts and whole cells. It is difficult to compare the data on MMO activitiy of different cell extracts because extraction, storage or protein concentration differ. As shown in Table 3.5.1, MMO activity in cell extracts are quite variable ranging from 8 - 175 nmol PO produced/min/mg protein (mU/mg protein). There are fewer reports of whole cell activities but again the methods of analysing the activitiy are different in each report. Furthermore, it appears that most methods have not been optimized until the report of Prior and Dalton (1985a). The highest whole cell MMO activity was 175 mU/mg cells demonstrated by Prior and Dalton (1985a). Whether this value is high enough for the commercial production of PO is dependant on other factors such as electron donor efficiency and stability of cells. It may be possible to compare this process with other biologically produced bulk chemicals. Ethanol and acrylamide are cheap biological products. Acrylamide has recently commercialized by Nitto chemicals (Yamada, 1988).

In the case of these bulk chemicals, the productivity (or cell activity: nmol/min/mg cells) is more than 90 mU/mg cells for ethanol (Lloyd and James, 1987) and more than 500mU/mg cells for acrylamide (Asano et al., 1982). The extremely high productivity of acrylamide production can be obtained because the reaction is a simple hydration, without any co-factor requirement. The 175 mU/mg cells of whole cell

Table 3.5.1

MMO activity in cell extracts

Methanotrophs			Specific activity (nmol/min/mg protein)	Reference
Type I				
Methylomonas	*methanica*	S1	22	1
M.	*methanica*		81	2
M.	*albus*	BG8	12	1
M.	*sp.*	CRL-17	8	1
Methylobacter	*capsulata*	Y	12	1
M.	*sp.*	23	20	1
M.	*sp.*	M26	10	1
Methylococcus	*capsulatus*	Bath	176	4
M.	*capsulatus*	Bath	83	2
M.	*capsulatus*	CRL-M1	42	1
Type II				
Methylocystis	*parvus*	OBBP	13	1
M.	*sp.*	CRL18	12	1
Methylosinus	*trichosporium*	OB3b	30	1
M.	*trichosporium*	OB3b	53	2
M.	*sporium*	5	17	1
M.	*sp.*	CRL15	68	5
M.	*sp.*	CRL15	37	1
M.	*sp.*	CRL31	75	3

References 1. Hou *et al.* (1980); 2. Dalton (1980);
3. Hou *et al.* (1982); 4. Prior and Dalton (1985a);
5. Patel *et al.* (1979)

MMO activity is not too far from the ethanol production rate but, taking into account the disadvantages of PO toxicity at low concentrations and instability of cells, the cell activity should be increased further to reduce the capital and recovery costs.

At the beginning of this project, Stanley (unpublished data) had succeeded in obtaining high MMO activity in whole cells (approximately 500 mU/mg cells) with Methylococcus capsulatus (Bath) from cultures at low biomass concentrations by optimising the assay conditions.

3.5.2 The Whole Cell MMO Activity of Methanotrophs

Cells were cultivated as described in Materials and Methods. Whole cells MMO activity (Wca) by various methanotrophs grown on NMS medium are shown in Table 3.5.2. Most of the organisms which were cultivated under low biomass conditions (less than OD_{540} = 8) showed relatively higher activities than earlier reports (See Table 3.5.1) Methylococcus capsulatus (Bath) exhibited more than 500 mU/mg cells and this value was 3 fold higher than that of recorded by Prior and Dalton (1985a).

It was sometimes observed in this study that even using the same NMS medium, the biomass concentration affected the Wca. Stanley et al. (1983) and Prior and Dalton (1985a) reported that the copper concentration affected the location of the MMO and MMO activity of Methylococcus capsulatus (Bath). Fig 3.5.1 shows the effect of copper concentrations on Wca of three methanotrophs. When the relative copper concentration (nmol copper/mg cells) in the culture is low, all of these organisms revealed low Wca. The Wca could be increased by the increasing the relative copper concentration. When relative copper concentration reached 3 nmol/mg cells no further increase was observed in Methylocystis parvus (OBBP), however in Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b), Wca still increased up to

Table 3.5.2

Whole cell activity of methanotrophs

Strain			Type	Whole cell activity (nmol/min/OD_{540} = 6)
Methylomonas	methanica	A4	I	205
M.	methanica	S1	I	520
M.	agile	A20	I	405
M.	rubra		I	410
M.	albus	BG8	I	495
M.	sp.	F13	I	365
Methylobacter	capsulata	Y	I	310
Methylocystis	parvus	OBBP	II	560
Methylosinus	trichosporium	OB3b	II	386
M.	sporium	12DF	II	403
M.	sporium	BGA	II	422
M.	sporium	5	II	415
Methylococcus	capsulatus	Bath	I (x)	513
M.	capsulatus	Texas	I	416

Whole cell activity was measured at 45°C.

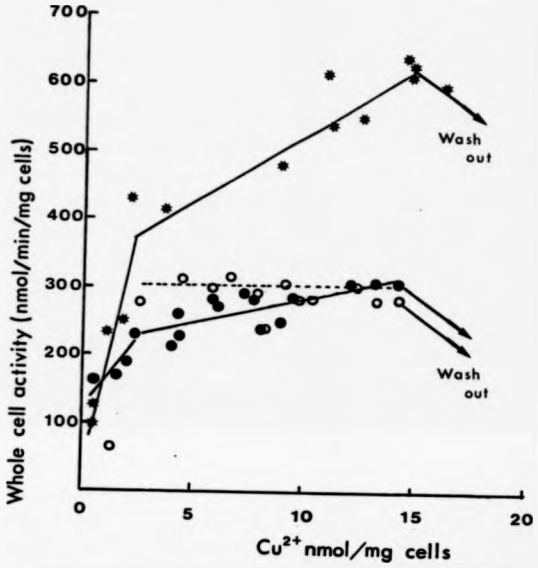


Fig. 3.5.1 The effect of copper concentration on whole cell MMO activity

Methylococcus	capsulatus	Bath	✱
Methylocystis	parvus	OBBP	○
Methylosinus	trichosporium	OB3b	●

a certain level. When copper was in excess, cells washed out (more than 15nmol/mg cells. By controlling the relative copper concentration, high MMO activity cells can therefore be obtained.

The other parameter to reduce the capital cost is to increase the biomass concentration in the reactor. Increasing the biomass concentration in the reactor would push up the net productivity if no limitation in reaction occurs. This means that a smaller bioreactor could be used for the same overall production rate.

By controlling the relative copper concentrations and dilution rates, high biomass cultivations (more than 8 g/l) were achieved which maintained a high activity in cultures of Methylococcus capsulatus (Bath), Methylocystis parvus (OBBP) (Table 3.5.3.).

3.5.3 Discussion

Most of the methanotrophs tested showed relatively higher activity than reported in the literatures. Two reasons can be considered responsible for this.

1) Cells had high activities but the methods used to determine whole cell MMO activity were not optimized.

   i) Biomass concentration
   Excessive biomass concentrations caused oxygen or electron donor limitation.

   ii) Electron donor concentration
   Each electron donor has an optimal concentration (Stanley, unpublished data) and both lower and higher concentrations

Table 3.5.3

Relationship between biomass concentration

and whole cell MMO activity

Strain	Biomass concentration (g/l)	$CuSO_4.5H_2O$ concentration (mg/l)	Whole cell activity (nmol/min/mg cells)
M. capsulatus (Bath)	1.82	0.2	101
	1.75	0.5	232
	1.88	1.0	431
	2.60	4.0	452
	3.67	10.0	610
	5.10	18.5	636
	8.91	18.5	596
	8.75	4.0	253
	8.50	1.0	131
M. parvus (OBBP)	0.90	0.2	64
	2.52	1.0	277
	3.27	1.0	305
	7.20	10.0	240
	9.30	20.0	279

Whole cell activity was measured at 45°C.

decreased the activity, especially high concentrations of methanol which inhibit MMO activity (Fig. 3.5.2).

iii) Assay period

When the assay period is long, the specific activity becomes lower due to inactivation of cells or electron donor- or oxygen-limitation.

iiii) Others

Assay apparatus, shaking speed, gas mixture ratio or temperature (Fig. 3.5.3) strongly effect on the Wca.

2) Low cell activities can be attributed to unsuitable culture conditions. It appears that both reasons are responsible for the low activities.

3.6 Final Comments

As indicated in the General Introduction, many PO producers have been found and investigated. However, little attention has focussed on which organism is better for the production of PO. PO producers can be classified into three groups. The characteristics for these organisms with their limitations are summarised below.

GROUP I Hydrocarbon-oxidising bacteria.

(1) Nocardia corallina B276 (Furuhashi, 1986)

a) productivity is low (15 mU PO/mg cells)

b) product yield is not high (PO is further metabolized)

c) glucose is an electron donor (1g PO/1.3g glucose)

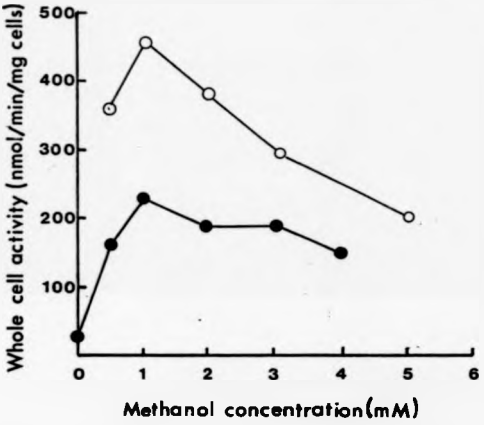


Fig. 3.5.2 The effect of methanol concentrations on whole cell MMO activity

○ Methylococcus capsulatus (Bath)

● Methylocystis parvus (OBBP)

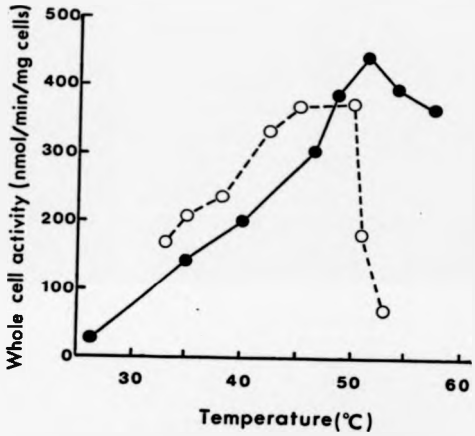


Fig. 3.5.3 The effect of temperature on whole cell MMO activity

Methylococcus	capsulatus	Bath	●
Methylocystis	parvus	OBBP	○

(2) Mycobacterium (Habet-Crützen et al., 1984;
Habet-Crützen and de Bont, 1987)

a) productivity is low (5-10 mU PO/mg protein)

b) product yield is not high (PO is further metabolized)

c) ethanol or ethane are electron donors (1 mole PO/1 mole ethanol)

GROUP II Ammonia-oxidising bacteria (Hyman and Wood, 1984)

a) productivity is high (250 mU ethylene oxide/mg protein)

b) product yield is high

c) hydrazine, hydroxylamine or ammonia are electron donors (1 mole ethylene oxide/1 mole ammonia)

d) difficult to grow high cell biomass

GROUP III Methane-oxidising bacteria

a) productivity is high (500 mU/mg cells)

b) product yield is high

c) methanol, methane or hydrogen act as electron donors.

Furuhashi (1986) suggested that the growth rate of micro-organisms on hydrocarbons is generally lower than on carbohydrates. Nocardia corallina (B276) is more advantageous than other hydrocarbon-oxidising organisms from the technical and economical point of view, because this organism can also grow on carbohydrates. Biomass concentration of Nocardia corallina (B276)in the culture was 15 g/l. However, this amount of biomass is not difficult to obtain in methanotrophs (30 g/l, see Chapter 10).

In the case of Mycobacterium, the electron donor system also has serious limitations, because ethanol is too expensive as an electron donor. Although ethane would be much better, its efficiency as an

electron donor is too low to be realistic possibility. In 1980, Drozd mentioned a surprising discovery that Nitrosomonas europaea could oxidise propylene, benzene or cyclohexane via the ammonia monooxygenase. This characteristic is similar to that of MMO and recently a wider specificity range has been recognised (Hyman and Wood, 1983; Hyman et al., 1985).

The productivity and yield of ethylene oxide by ammonia-oxidising bacteria closely resembled the properties of methanotrophs. For the production of PO, ammonia-oxidising bacteria required a reduced nitrogen compound, such as hydrazine or hydroxylamine as electron donors but unfortunately these compounds are more expensive than PO, and are not economically suitable. Ammonia also act as an electron donor, but when ammonia was used as a donor, the productivity dropped from 250mU ethylene oxide produced/mg protein to 40 mU ethylene oxide produced/mg protein. The price of ammonia per kg is nearly double that of methanol, but the molecular weight is approximately half of methanol, so the relative costs are similar. If productivity, electron donor efficiency and cell-growth can be improved, ammonia-oxidising bacteria could become a strong competitor to methanotrophs for possible industrial PO production. One problem is that only ethylene oxide production was investigated by Hyman and Wood (1984) and no information on propylene oxidation was provided. At present, it was concluded that methanotrophs were the best organisms for the production of PO.

CHAPTER 4

Stability and Inactivation of Methane Monooxygenase in Whole Cells

4.1 Introduction

These are three important factors to consider in the development of PO production technology using micro-organisms. The first is the activity of cells as mentioned in Chaper 3 and the second is the stability of PO production activity; not only for MMO activity but also for the electron donor system. The third factor is the stoichiometry, that is, how much PO can be produced from one unit (mole) of electron donor (methanol).

The investigations on the stability of soluble and particulate MMO obtained from Methylococcus capsulatus (Bath) (Colby and Dalton, 1976, 1978; Prior, 1985) or Methylosinus trichosporium (OB3b) (Stirling and Dalton, 1979a; Pilkington, 1986) inform us that cell extracts are not suitable as biocatalysts for the production of PO. Hence whole cells are necessary as MMO in cell extracts is unstable at the optimal temperature for PO production. Nevertheless, information on the stability of whole cells is extremely limited. Droxed et al., (1978) studied the stability of whole cells of Methylococcus sp. NCIB 11083 by measuring the specific lysis rate. They found that cell lysis was affected by the dilution rate of culture i.e. cell lysis was increased by increasing the dilution rate.

Subramanian (1986) noted that cell suspension of Methylococcus capsulatus and Methylosinus trichosporium could be stored for 10 days at 24°C and up to 3 weeks at 4°C with little loss of activity. In this Chapter, stability of whole cells under different conditions are investigated.

4.2 The Stability of Cells under the Conditions of Non-PO Production

The loss of catalytic activity by methanotrophs during storage may

provide important information about the loss of activity during the PO production.

4.2.1 Experimental

The cultures were collected from a steady state culture (0.7l fermenter, dilution rate D = 0.1hr^{-1}) and were centrifuged once. The cells were then suspended in phosphate buffer or NMS medium at a final biomass concentration of OD_{540} = 6. Twenty five millilitres of cell suspensions were kept under different storage conditions without carbon source and the time course of inactiviation of whole cells was monitored. Methylococcus capsulatus (Bath), Methylocystis parvus (OBBP) and Methylosinus trichosporium (OB3b) were used for the stability test.

4.2.2 Results

4.2.2.1 Cells

To compare the stability of the biocatalytic activity cultivated under the conditions of soluble-MMO cells and particulate-MMO cells of Methylococcus capsulatus (Bath), both types of cells were kept in shaking flask aerobically. Fig 4.2.1 shows the decay of whole cell activity which were measured using 1mM methanol as a donor in assay. Particulate-MMO cells were found to be twice as stable as soluble-MMO cells under these conditions.

4.2.2.2 The Effect of Temperature on the Stability of Particulate MMO Cells

The loss of catalytic activity in extracted MMO was strongly

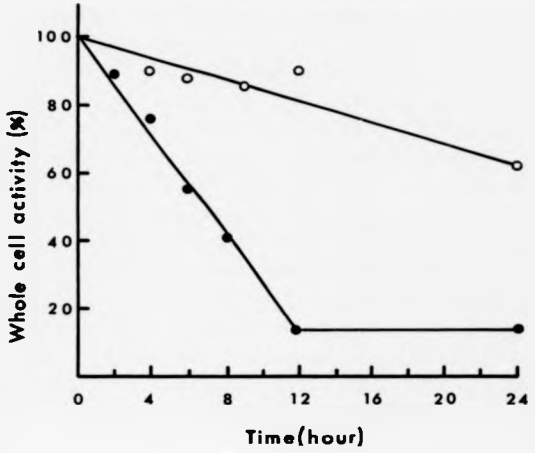


Fig. 4.2.1 The stability of the cells under conditions of non-PO production

○ Particulate-MMO cells

● Soluble-MMO cells

Cells were kept at 45 °C aerobically.

dependent on the temperature (see Chapter 3). This relation was also seen in the case of whole cells, but the rates of decay were much less than that of extracted MMO's.

It was suggested that oxygen might affect the stability of Wca as well as temperature. To test this, particulate-MMO cells were kept under anaerobic conditions. Under these conditions it was found that the cells could be stored for one week with a little loss of activity (Fig. 4.2.2). Similar temperature and oxygen effects were also observed in Methylosinus trichosporium (OB3b) and Methylocystis parvus (OBBP).

As reported here, increasing the temperature above 30°C accelerated the decay of MMO activity, especially when the temperature exceeded the maximum growth temperature of the organism, therefore this must be avoided. The medium composition is also important and the decay of activity was accelerated when cells were kept in phosphate buffer compared with storage in NMS medium (Fig 4.2.3) at 45°C in Methylococcus capsulatus (Bath). Accordingly, the cells should be kept in growth media and not simple buffer solutions such as phosphate buffer.

The fact that whole cell activity showed greater stability under anaerobic conditions further complicates PO production, as an oxygen supply is necessary for PO production. Therefore dissolved oxygen control will play an important role in keeping the oxygen concentration in a system at its optimum for PO production.

4.3 The Effect of PO Concentration of the Growth of Methylococcus capsulatus (Bath) and Methylocystis parvus (OBBP)

The aim of this experiment was to determine at which exogenous concentrations of PO was cell function impaired. Growth inhibition in the batch culture was used as one of the tests of the toxicity of the propylene oxide. Continuous cultures were grown in 700ml fermenters

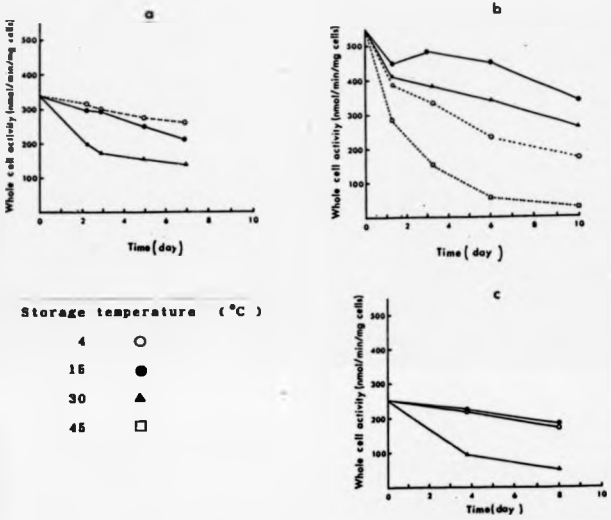


Fig. 4.2.2 The stability of cells under anaerobic conditions

a *Methylocystis parvus* (OBBP)

b *Methylococcus capsulatus* (Bath)

c *Methylosinus trichosporium* (OB3b)

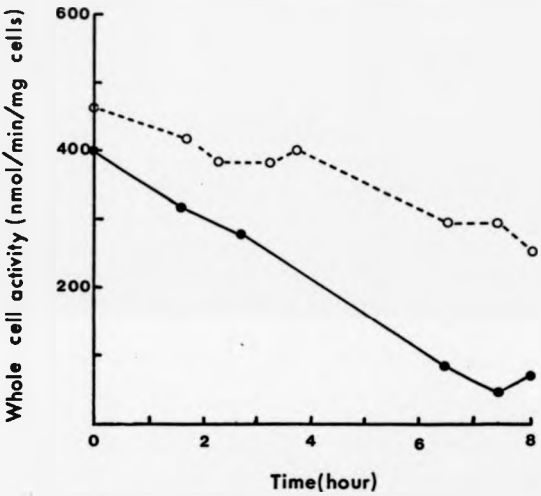


Fig. 4.2.3 The decay of whole cell activity during storage in phosphate buffer under aerobic conditions

● Phosphate buffer

○ NMS medium

(New Brunswick Scientific, edison, N.J, U.S.A) using methane as a carbon source. Methylococcus capsulatus (Bath) and Methylocystis parvus (OBBP) were cultivated continuously and were used as inoculant when the OD_{540} was 16 and 15 respectively. 0.5ml of culture was taken from the continuous culture and inoculated to 250ml flasks each of which contained 25ml of NMS medium. One hour after inoculation PO was added to give theoretical concentrations (0.12 - 1.0mM) in each flask. Further growth was followed turbidometrically. Results are shown in Table 4.3.1.

No significant inhibition of growth was detected in flasks containing dissolved concentrations of 0.2mM PO or lower in Methylococcus capsulatus (Bath) after 7 hours. Higher dissolved concentrations (0.4mM) produced a marked decrease in growth rate. Methylocystis parvus (OBBP) would tolerate concentrations up to 0.4mM. The concentration at which growth rate was affected for Methylocystis parvus (OBBP) was shown to be 0.6mM.

4.4 Inactivation of Cells under the Conditions of PO Production with PO Accumulation

As mentioned above, the catalytic activity of methanotrophs is fairly stable when in an anaerobic environment at temperatures between 4 - 20°C. However, cells are not stable when PO is present in the culture. The most important thing for the development of the PO production technology is to stabilise the biocatalyst by elucidating the inactivation mechanism under the conditions of PO production. To understand the relationship between PO production and inactivation, PO was produced endogenously using fermenter cultures.

Table 4.3.1

Effect of PO concentration in the culture on cell growth

Organism	Initial PO concentration (mM)	Cell growth *) (OD_{540})
M. capsulatus (Bath)	0	0.786
	0.12	0.762
	0.20	0.701
	0.43	0.360
	0.68	0.292
M. parvus (OBBP)	0	0.612
	0.14	0.508
	0.25	0.504
	0.41	0.498
	0.59	0.304
	0.81	0.306
	1.02	0.286

*) Cell growth was measured 7 hours after PO was added.
Initial OD_{540} were 0.302 in Methylococcus capsulatus (Bath) and 0.278 in Methylocystis parvus (OBBP).

4.4.1 Experimental

Cultures of Methylococcus capsulatus (Bath) containing particulate MMO were collected from an oxygen-limited steady state ($D = 0.1hr^{-1}$) culture (10 L fermenter) and diluted with NMS medium (copper concentration 0.5ug/l). The cell suspensions were placed into a 700ml fermenter. Typically, the fermenter was filled with 400ml of cell suspensions. A gas mixture of propylene and air or propylene, air and methane was supplied. Methanol was supplied simultaneously as an electron donor. The PO concentration in both the gas and liquid phase were analysed periodically using gas chromatography. The decay of whole cell activity was also analysed after removing PO from culture samples by scrubbing with air. Following the addition of propylene and methanol, PO was produced and accumulated in the reaction mixture. The rate of PO production was quantitated from the amount in both the gas and liquid phase. The biomass concentrations were also varied, but specific methanol addition rate (nmol methanol/min/mg cells = mU/mg cells) was kept at a constant rate of 200 mU/mg cells.

4.4.2 Results

The three experimental results are shown in Fig 4.4.1, 4.4.2, 4.4.3. PO concentration in the reaction mixture dramatically changed within a few minutes as seen in Figure 4.4.1. During this period the cells lost their activity very rapidly. However the inactivation rates were independent of PO concentration in the reaction mixture. The PO concentration produced by the dense culture (Fig. 4.4.2) was significantly higher than that produced by the lower biomass density (Fig. 4.4.3) but the inactivation rate of high biomass condition was less. The PO concentration of lower biomass culture reached at a level

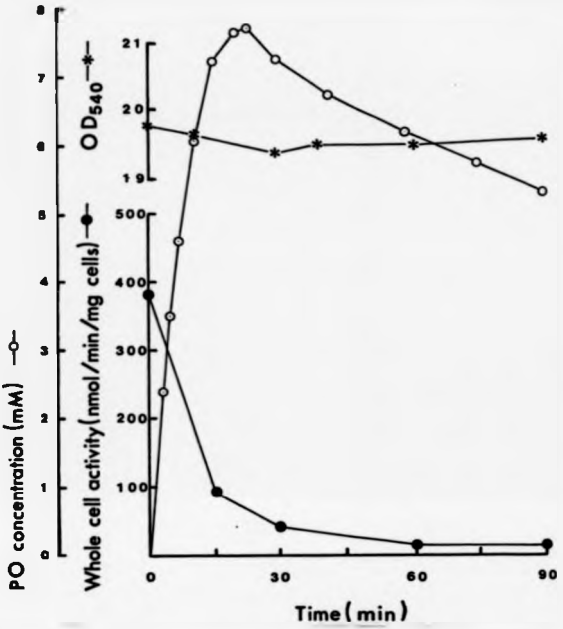


Fig. 4.4.1 Inactivation of cells under the condition of PO production with PO accumulation

High biomass - high concentration of PO accumulation

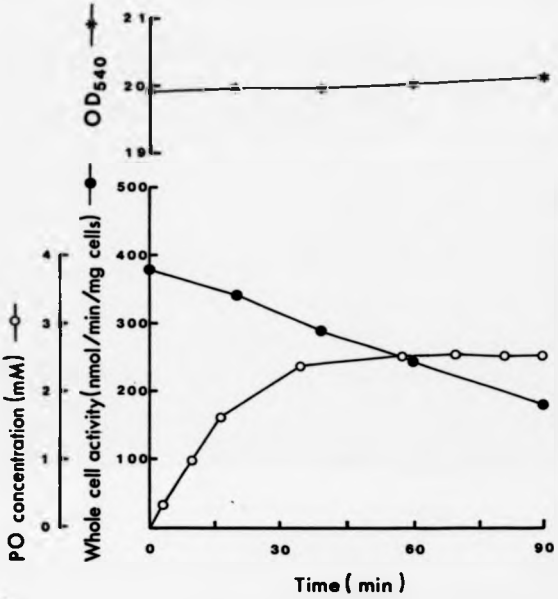


Fig. 4.4.2 Inactivation of cells under the condition of PO production with PO accumulation

High biomass - moderate concentration of PO accumulation

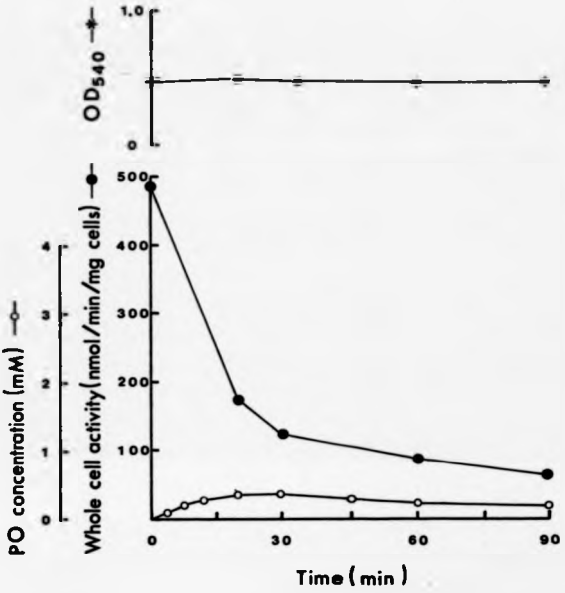


Fig. 4.4.3 Inactivation of cells under the condition of PO production with PO accumulation

Low biomass - low concentration of PO accumulation

of 0.3mM after 20 minutes (Fig. 4.4.3). This concentration would appear to be similar that observed to cause inactivation of Methylococcus capsulatus (Bath) in cell growth experiments (see last Section). Yet 0.3mM did show an inhibitory effect on activity of the cells.

It therefore seems that the inactivation rate and PO concentration are independent. In these experiments, both biomass and PO production rate were altered, but also probably oxygen tension in the reactor was different in each experiment. The PO production rates were found to be faster initially and then decreased gradually until complete inactivation occurred. Although this initial PO production rate was varied between experiments, it was higher in the low cell density conditions than the high cell density conditions. From these experiments, it was suggested that both oxygen tension and PO production rates affect the inactivation rate more strongly than bulk PO concentration per se.

4.5 Discussion

From the investigations of the stability of cells under non-PO producing conditions, oxygen appeared to be one of the causes for inactivation of cells. The effect of oxygen on the loss of whole cell MMO activity is probably not simple. Soluble MMO has an iron-sulphur centre in its protein. Turnbough and Switzer (1975a) reported that glutamine phosphoribosylpyrophosphateamidotransferase (amidotransferase) is regulated in Bacillus subtilis through an oxygen-dependent inactivation of the cells, in the stationary phase of growth. This enzyme has an iron-sulphur centre in its molecule. The nature of the oxygen-dependent inactivation of amidotransferase was clarified by reaction with oxygen in cell extracts (Turnbough and Switzer, 1975b) and the subsequent demonstration that pure amidotransferase is an oxygen labile iron-sulphur protein (Wong et al., 1977; Averille et al., 1980).

Inactivation is accompanied by bleaching of the iron-sulphur chromophore (Wong et al., 1977), loss of inorganic sulfide (Switzer et al., 1979 a,b) and conversion of the iron from a form assaying as Fe^{2+} to Fe^{3+} (Switzer et al., 1979a,b). No specific enzyme was required to inactivate amidotransferase. The instability of soluble MMO may be related the oxidation of the iron-sulphur centre by oxygen.

There are several factors which play a role in the loss of activity in the presence of PO. These factors are PO concentration, dissolved oxygen, electron donor and PO production rate. Obviously some of these factors play a greater role in the inactivation of cells. Recently, Habet-Crützen and de Bont (1985) have demonstrated that PO added externally to a cell suspension of Methylosinus trichosporium (OB3b) inactivated propylene oxidising activity and they suggested that PO inactivated MMO irreversibly. Stanley and Richards (unpublished data) from this project obtained quite interesting results on the inactivation of cells by exogenously supplied PO. Their results are shown in Table 4.5.1. They noted that methanol alone, or PO alone were not as inhibitory (inactivating) as a mixture of methanol and PO. They also suggested that the presence of propylene protected the cells from inactivation by PO. Argon in place of propylene did not show any protection effect.

Habets-Crützen and de Bont (1985) concluded that Methylosinus trichosporium (OB3b) was inactivated by external PO to 50% of the initial activity at concentrations of approximately 3mM whatever the conditions. The experimental conditions were not shown clearly, but they did not add any electron donors. However, the results obtained by Stanley and Richards show that the concentration of PO which produced 50% inactivation may well be dependant on the condition of the electron donors, which may be either exogenous or endogenous.

Table 4.5.1

Inactivation of whole cells by PO externally added

in Methylococcus capsulatus (Bath)

Reaction mixture	Wca (% activity remaining after 10 minutes)
Methanol	100
Propylene + PO	94
PO	56
Propylene + Methanol (PO production)	37
PO + Methanol	13

Methanol - 600 nmol/min/mg cells, feed started at zero time

Propylene - 20% in air

PO - 2.8 mM PO added at zero time

Reaction condition; Temperature : 45°C, Biomass 1g dry cell/l
Agitation : 600 rpm

Data was taken from Stanley and Richards (unpublished data)

The effects of PO on the inactivation of whole cell MMO activity which have been demonstrated by several workers and examined in this thesis are summarized below.

a) The PO concentration affects the growth of methanotrophs which is particularly marked at high concentrations of PO in the medium where a dramatic decrease in the growth rate is observed. (Stanley and Richards, unpublished data; this thesis).

b) Higher dissolved concentrations of PO inactivate the biocatalyst (Subramanian, 1986). PO concentration in the reaction mixture also strongly affects the inactivation of cells of Nocardia (Furuhashi et al., 1981) and Mycobacterium (Habets-Crützen and de Bont, 1985)

c) Under the PO production conditions, the effect of PO accumulated in the reaction mixture on the loss of Wca is variable. Even at concentrations of PO which do not affect the growth of cells, rapid inactivation occurs (this thesis).

d) Oxygen and electron donors accelerate the inactivation of cells by externally added PO and propylene protects the loss of activity (Stanley and Richards, unpublished data; Miyazaki et al., 1986 using Nocardia).

The findings of Stanley and Richards are most impressive. They found that the inactivation of cells by exogenously-supplied PO required both oxygen and electron donor. Furthermore, propylene protected the inactivation by PO under conditions of non-PO production (no electron donor and/or oxygen supply). These phenomena support the hypothesis that the PO which is added exogenously inactivates the active centre of

the MMO. Prior and Dalton (1985b) reported that the partial inactivation of soluble MMO activitity occured in the presence of acetylene alone or acetylene plus either oxygen or NADH.

Total inactivation of MMO activity, however, required the presence of both NADH and oxygen and was due to the enzyme catalysed oxidation of acetylene to ketene which irreversible bound to neighbouring amino acids (see General Introduction). The results were also shown for MMO activity in the particulate fractions of cell extracts and were similar to the soluble system. Prior and Dalton (1985b) also showed the [^{14}C]-labelled acetylene bound to soluble MMO and suggested that acetylene was a suicide substrate of MMO.

At no time during any of the assay procedures reported here was a product of PO oxidation detected by gas chromatography suggesting that the oxidation of PO may lead to formation of an enzyme complex which has no further MMO activity. This suggested that propylene was not a suicide substrate but PO may be a suicide substrate of MMO like acetylene.

Finally, it becomes clear that the time required for the loss of 50% of the initial activity (half life) is extremely short. A half life of less than 15 minutes under the conditions of PO production has been observed (see Fig. 4.4.1). This short half life of whole cells is not far from the half life of extracted MMO. Under these situations, the development of PO production technology from laboratory scale to more advanced scale is impossible without an increase in longevity.

It has been suggested that the PO concentration in the reaction mixture is a major source of inactivation (Subramanian, 1986; Furuhashi et al., 1981; Habets-Crützen and de Bont, 1985), if this is so the loss of activity could be prevented by the rapid removal of PO from the reaction mixture. Alternatively, if PO production rate per se the major reason for inactivation then loss of activity would not be affected by

PO concentrations. To elucidate the inactivation mechanisms under the conditions of PO production, the new system 'immobilized cell-biofilm reactor' was designed. This immobilized cell-biofilm reactor makes it possible to evaluate the effect of external PO concentrations on inactivation under both conditions of PO production and non-PO production.

CHAPTER 5

Inactivation of Whole Cells under the Conditions of PO Production without PO Accumulation

5.1 Introduction

Many workers have been endeavouring to remove PO which is accumulated in the reaction mixture following the oxidation of propylene. Furuhashi et al. (1981) used the agitation-type reactor for the production of PO by Nocardia corallina (B276). He noted that the production rate was increased by reducing the PO concentration in the reaction mixture by increasing the reaction gas flow rate. Miyawaki et al. (1986) have also demonstrated an improvement in the productivity of PO by increasing the gas flow rate using a gas flow bubble reactor which was packed with immobilized cells of Nocardia corallina (B276).

Hou (1984b) designed the gas-solid bioreactor to improve the stability of cells and productivity of PO production using Methylosinus sp. CRL-31. A similar method was also applied by Habets-Crüzen and de Bont (1987) for the production of PO by alkene-oxidising Mycobacterium. In spite of these investigations, the stability of cells and productivity were not improved significantly.

The concentration of extracellular PO might affect inactivation. To investigate the effect of external PO concentration on the inactivation, it is necessary to keep the PO concentration in the reaction mixture constant. However, this is very difficult to achieve if an ordinary agitation-type reactor is used, because the PO concentration increases during the biotransformation and therefore makes interpretation of the results difficult (See Fig. 4.4.1).

To investigate the effect of PO concentration on the inactivation of the biotransformation, it was therefore necessary to design an immobilized cell-biofilm reactor (IBF-reactor) to facilitate these studies. Such a reactor has the advantage that any PO produced could be immediately removed from the vicinity of the organisms and permit

determination of the direct effect of externally supplied PO in the system.

5.2 The Optimization of Reaction Condition of IBF-reactor

Cells containing particulate MMO of Methylococcus capsulatus (Bath) were used in this Section. Before studying the effect of PO concentration on the stability of cells, it was necessary to compare the productivity in the IBF-reactor with the whole cell activity (Wca) to determine whether PO production rate in the reactor was a genuine reflection of cellular capacity, or was limited by abiotic variables. From preliminary experiments it was found that PO productivity in the IBF-reactor was affected by the gas composition, electron donor, biomass and medium flow rate. Fig. 5.2.1 shows the time course of PO production in the IBF-reactor. Immediately after the reaction was started, the PO concentration in the outlet medium increased and usually reached a maximum value after 1 - 3 minutes. The PO productivity which shows a maximum during the reaction is called the 'peak productivity'. The value of this peak productivity is similar to that of Wca measured separately. After peak productivity appeared, PO production rapidly decreased. The effect of reaction conditions on PO productivity are summarized below.

a) When air was used as an oxygen source, the peak productivity was limited by oxygen supply compared to the peak obtained using pure oxygen. Accordingly, the use of pure oxygen was preferable for the IBF-reactor (Fig 5.2.2).

b) The peak productivity was limited by electron donor availability below 0.3mM methanol and methanol concentrations in excess of 1mM

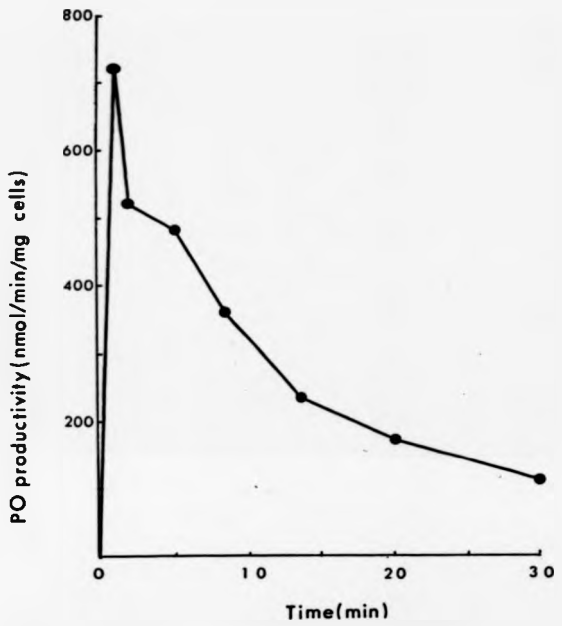


Fig. 5.2.1 The time course of PO production in the IBF-reactor

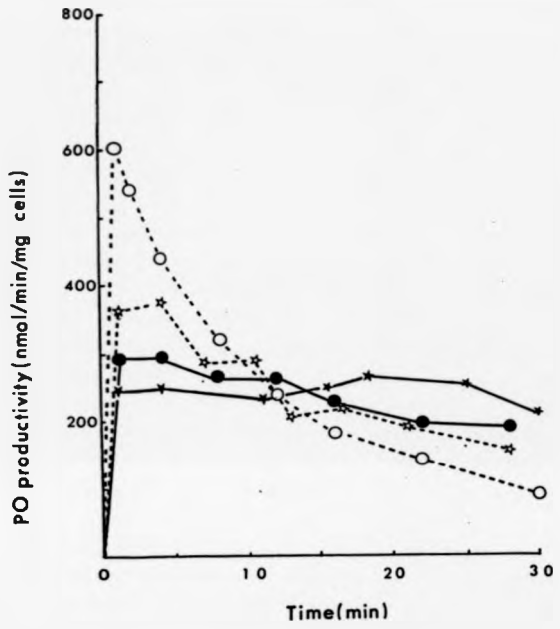


Fig. 5.2.2 The invisible inactivation during PO production under limiting conditions in the IBF-reactor

Methanol concentration (mM)	Gas composition PP : O_2
✱ 0.1	1 : 2
✱ 0.3	1 : 2
○ 0.3	1 : 1
● 0.3	1 : 1

depress the peak productivity due to the competitive inhibition exerted by methanol on MMO. Routinely concentrations ranging from 0.3 to 0.5mM methanol were used (Fig. 5.2.3).

c) The total biomass on the filter also influenced the productivity. By increasing the biomass loading, the oxygen, substrate and electron-donor requirement increased correspondingly. Furthermore pressure at the inlet was increased by increasing the biomass on the filter and this might affect the productivity. Moreover, low levels of biomass on the filter caused a decrease of PO concentration in the outlet to a level too low to permit accurate quantification. From these studies it was determined that from 0.5 - 2.5mg dry weight of cells were optimum for the 4.9 cm^2 surface of filter.

d) Medium flow rate also affected the productivity for the reasons similar to those above. Flow rates of 2 - 4ml/min were routinely employed for optimum productivity.

Optimization of gas composition, methanol concentration and media flow rate enabled a productivity of more than 700 mU/mg cells to be achieved in the IBF-reactor with Methylococcus capsulatus (Bath). This productivity is the highest recorded value for the production of an epoxide by micro-organisms.

The peak productivity of the IBF-reactor always gave a higher value than the Wca of the same culture measured in the standard 3 minutes flask assay. The difference between these two values are considered to be due to the following reasons.

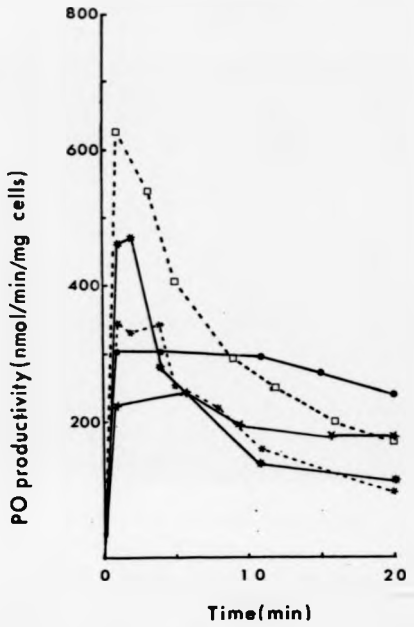


Fig. 5.2.3 The effect of methanol concentration on the peak productivity in the IBP-reactor

Methanol concentration (mM)

- ● 0.1
- □ 0.3
- ● 1.0
- ✱ 1.5
- ✱ 5.0

1) In the 3 minutes shake flask assay only PO produced in the liquid phase is measured, PO in the gas phased being discounted, consequently the actual activity is higher than Wca so calculated.

2) The peak productivity in the IBF-reactor is an instantaneous value whereas the Wca in shake flasks is a mean value over three minutes. During this time PO producing activity may change drastically.

3) In the IBF-reactor, the reaction conditions are kept constant under optimal reaction conditions throughout the reaction period, however in shake flask, the conditions change progressively, especially the electron donor concentration and PO concentration, maintaining constant optimum conditions are therefore impossible.

4) Even within three minutes the PO accumulated in the flask may accelerate the inactivation and/or inhibition of cells activity in the shake flask.

However, these two numbers, that is peak productivity and Wca are quite similar, and this evidence suggests that peak productivity in the IBF-reactor represents the genuine cell activity if the reaction is not limited by oxygen, electron donor or propylene.

5.3 Interpretation of Kinetics of Inactivation on Immobilized Cell-biofilm Reactor

Throughout the experiments on the optimization of conditions, one important feature concerning the peak productivity and inactivation was found. The peak productivity in the IBF-reactor was depressed under oxygen- or electron-donor limited conditions compared to the non-limited

condition. However, little or no inactivation was observed during the early phase of the reaction under these conditions. It is possible that invisible inactivation of cellular enzymes occurred, becoming apparent only when the whole cell activity fell so low that the PO production was no longer limited by the concentration of substrates.

As shown in Fig. 5.3.1 under electron donor-limited conditions (0.05mM methanol), initial productivity was low, but when the methanol concentration was increased to 0.3mM the productivity increased to the potential cell activity and then progressively decreased potential cell activity as a result of inactivation.

This phenomenon was also observed when methanol was in excess. Methanol is a competitive substrate of MMO (Colby et al., 1977). It is possible therefore that when the methanol concentration was high, oxidation of propylene was reduced. In this state, the productivity in the IBF-reactor does not represent maximum cellular activity.

5.4 Use of the IBF-reactor

5.4.1 Comparison of Peak Productivity and Whole Cell Activity

So far, PO accumulated or added to the reaction mixture has been postulated to inactivate cells. However, suprisingly, in the IBF-reactor, cells lost activity quickly even through PO was rapidly removed and did not accumulate. This loss of activity must be reflected in the cell activity. To test this result, cells were collected from the surface of the filter after 30 minutes reaction by flushing back through with NMS medium for the outlet side. Cells were concentrated by centrifugation and resuspended in NMS medium. Then the Mca was measured by the shake flask method. The same experiments were repeated 5 times and mean values were calculated. The initial activities measured in the

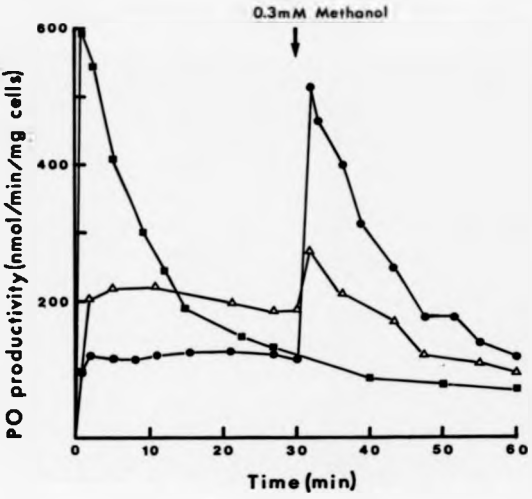


Fig. 5.3.1 The invisible inactivation during PO production under limiting conditions in the IBF-reactor

Methanol concentration (mM)

● 0.05

▲ 4.0

■ 0.3 (Control)

shake flask and IBF-reactor (peak productivity) were 420 and 606 mU/mg cells respectively. The residual activities were 101 and 112 mU/mg cells respectively. From these results, the decrease of productivity in the IBF-reactor was confirmed as responsible for the loss of cell activity. Therefore some unknown inactivation mechanism other than external PO was thought to exist under conditions of PO production and this inactivation was more severe than that exerted by PO added exogenously.

In order to investigate whether the rapid inactivation is dependent on reaction conditions or not, effects of physical parameters such as pH, temperature on the inactivation were studied.

5.4.2 Effect of pH on the Inactivation

Propylene oxide is a chemically reactive compound and is quite labile at low and high pH values where it is rapidly hydrolized to propylene glycol. The effect of pH value on the inactivation under the PO production conditions was investigated.

The cells were deposited on the filter and were washed with 20mM phosphate buffer at pH values of 6, 7, 8 and 8.5. To date, NMS medium was used for the studies in the IBF-reactor but to avoid the precipitation of elements under alkaline conditions, phosphate buffer was used in this experiments. The filters were kept under different pH conditions for 5 minutes, then the reactions were initated by changing buffer to one which contained propylene, oxygen and methanol.

Results are shown in Fig 5.4.1. No relationship was observed between inactivation rates and the pH of the reaction mixture during the 30 minutes reaction period. Methylococcus capsulatus (Bath) grows within a range of pH values 6 to 8.5, however at pH 6 or 8.5 growth is depressed and cells grown under these conditions show low MMO

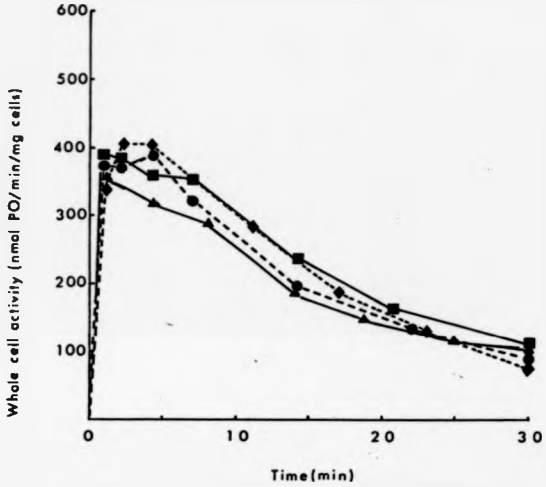


Fig. 5.4.1 The effect of pH on the inactivation of cells in the IBF-reactor

pH ▲ 6.0
■ 7.0
● 8.0
◆ 8.5

activities. Accordingly, the pH value may affect cell activity over a long period but rapid inactiviation of PO production was independant of pH.

5.4.3 Effect of Temperature on the Inactivation

It is well known that temperature affect enzyme activities. Therefore it was considered that temperature affected inactivation of cells. The effect of temperature on the Wca measured in shake flasks has been shown before (see Fig. 3.5.3). The highest activity in Methylococcus capsulatus (Bath) was found at 50°C. The cells cannot grow at this temperature (maximum growth temperature: 47°C). When the reaction temperature in the IBF-reactor was lowered, the peak productivity was decreased (Fig. 5.4.2). At the same time the inactivation pattern was changed. The half life at 45°C and 40°C were about 6.5 and 7.5 minutes respectively. Following the decrease in temperature half lives were prolonged. At 35°C, the half life was 9.5 minutes and at 30°C, it was more than 30 minutes. The decrease in inactivation rate reflected the reaction temperature, however this phenomenon was considered to be responsible to the decrease of PO productivity.

5.4.4 Comments on Results Concerning the IBF-reactor

As mentioned so far, it appears difficult to prevent the inactivation following the PO production. It was considered that the inactivation influenced by the PO productivity. Fig. 5.4.3 shows the relationship between peak productivity and the time elapsed until the activity decreased to half of the peak activity. By limiting the electron donor supply, variable peak productivities could be obtained. These peak productivities did not represent the potential actvities, but

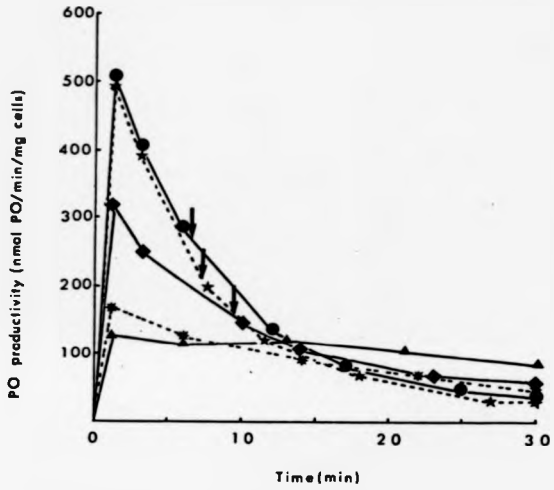


Fig. 5.4.2 The effect of temperature on the inactivation of cells in the IBF-reactor

Temperature
25 °C ▲
30 ✱
35 ◆
40 ✱
45 ●

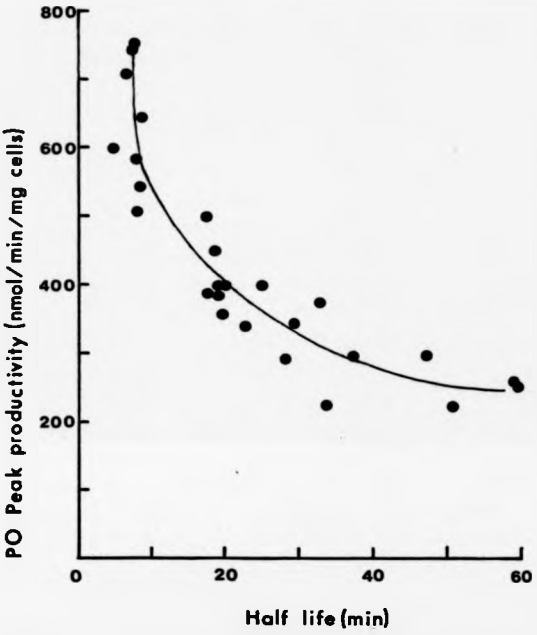


Fig. 5.4.3 The relationship between peak productivity and the time elapsed until the activity decreased to half of the peak activity

after a certain time, when cell activity was lost and the electron donor was no longer limiting, the productivity of the IBF-reactor and cell activity approached the same value of potential activity.

The relationship between peak productivity and half life indicates that peak productivity and/or the net PO produced affects the rate of inactivation of cells. By increasing the PO productivity to more than 200 mU/mg cells, inactivation was accelerated and the half life was shortened. 200 mU/mg cells of peak productivity may be a critical point for the other unknown inactivation mechanism. In the next Section, effects of external PO concentration on inactivation of cells under the conditions of PO production are studied.

5.5 Effect of Extracellular PO Concentration on the Inactivation under the Conditions of PO Production

The principal reason for developing the IBF-reactor was to investigate the effect of extracellular PO concentration on the inactivation of cells under the conditions of PO production. From the results obtained to date it has become clear that the inactivation appears to be due to the concerted effect between the external PO and an internal unidentified effect following the oxidation of propylene. To investigate the concentration of the extracellular PO concentration on the inactivation during PO production from propylene, PO was added to the reaction medium which also contained propylene, oxygen and methanol.

5.5.1 Experimental

To investigate the effect of external PO concentration, the reaction medium containing varied amounts of PO and also containing propylene, oxygen and methanol were supplied to the IBF-reactor for five

minutes. The reaction medium was then switched to the medium without PO. If PO which is added externally accelerated the inactivation, then the peak productivities are expected to decrease following the change over of medium which does not contain PO.

5.5.2 Results

The results are shown in Fig. 5.5.1. No significant effect of PO on acceleration of inactivation was observed by the treatment with 0 - 1.0mM PO for 5 minutes, but when cells were treated with 1.9mM PO under the conditions of PO production, inactivation was accelerated. The cells which were treated with PO concentrations ranging from 0 - 1.0mM lost about 100mU over the initial 5 minutes. This loss of activity was probably due to the oxidation of propylene. Although, the cells which were treated with 1.9mM PO lost approximately 200mU over 5 minutes. This difference of 100mU over 5 minutes appears to be an effect of the extracellular PO concentration.

In the above experiment, cells were treated with PO under the conditions of PO production for only 5 minutes. In order to investigate the effect of the treatment period on inactivation, cells were treated for 30 minutes with medium which contained varied amounts of PO and also contained propylene, oxygen and methanol. Losses of activity were calculated from the difference between concentration of PO in the inlet and outlet streams. Results are shown in Fig. 5.5.2. There was no significant difference in the ratio of inactivation between control (no PO) and up to 1.2mM of added PO. This suggests that external PO concentration is important but that more than 1.2mM PO is necessary to accelerate the inactivation of cells. Under low PO concentrations (up to 1.2mM), treatment periods of up to 30 minutes do not appear to cause any stimulation of inactivation rates. However the external PO was

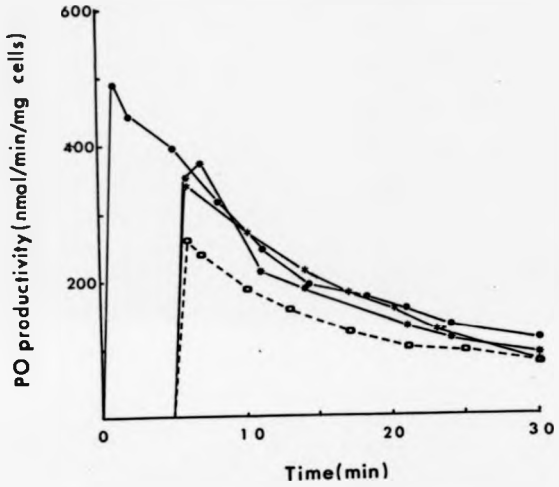


Fig. 5.5.1 The effect of the external PO concentration on the inactivation of cells

PO Concentration
(mM)
● 0
✱ 0.84
● 1.0
■ 1.9

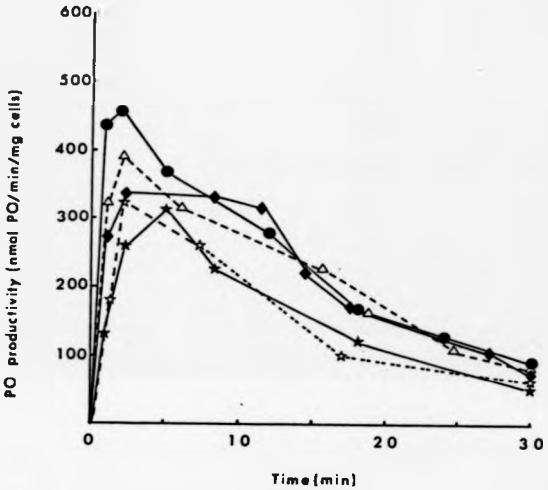


Fig. 5.5.2 The effect of the external PO concentration on the inactivation and on the productivity of the cells

PO concentration (mM)

- ● 0
- △ 0.25
- ◆ 0.56
- ★ 0.84
- ☆ 1.21

found to affect the peak productivity which was reduced by increasing the external PO concentration. This phenomenon supports the idea that PO is a substrate of MMO and competes with propylene (product inhibition).

5.6 Discussion

Throughout the experiments using IBF-reactor, it was assumed that three factors affected PO production:-

a) inactivation of cells following the oxidation of propylene to PO without external PO,

b) the acceleration of inactivation by PO which is accumulated externally,

c) the inhibition of PO production by competing with propylene and PO as the substrates of MMO.

The most disappointing result was the rapid inactivation of activity under the conditions of PO production without PO accumulation. This was the major cause of inactivation of the biocatalyst under high PO production conditions. This inactivation has not been reported previously, because workers have not examined the kinetics of the inactivation process under the conditions of PO production. The inactivation by this unknown effect associated with the oxidation of propylene was dependent on the peak productivity and/or net amount of PO produced and inactivation was accelerated by the increase of PO productivity.

On the other hand, the inhibition and inactivation were accelerated by increasing the extracellular PO concentration. It is clear that the PO concentration in the reaction mixture should be decreased to maintain biocatalyst longevity as pointed out by many workers (Furuhashi et al., 1981; Habets-Crützen et al., 1984; Subramanian, 1986). The extracellular concentration tolerated by Methylococcus capsulatus (Bath) is 1.2 - 1.9mM under the conditions of PO production. Under the conditions of non-PO production condition, PO in the cell suspension is capable of inactivating at much lower concentrations than under conditions of PO production. These differences appeared to be due to the protection effect of propylene. The affinity of propylene for MMO is higher than that of PO for the enzyme (Green and Dalton, 1986). Accordingly, the electrons are mainly used for the oxidation of propylene but not PO. A lower affinity for ethylene oxide than for ethylene in ammonia monooxygenase of Nitrosomonas europeae has been reported by Hyman and Wood (1984).

It is possible to reduce the inactivation effect of extracellular PO concentration by optimizing the reaction conditions e.g. control of PO, amount of electron donor, but it seems very difficult to prevent the inactivation following the oxidation of propylene. This inactivation rate is strongly dependent on the PO productivity, but less dependent on production conditions such as pH or temperature (temperature effects on productivity). Furthermore, it was found that the inactivation was less dependant on copper concentrations, electron donor species, and the cells which contained soluble MMO also inactivated rapidly under the conditions of PO production without external PO (these data are not shown).

Since inactivation of PO production is an inevitable consequence of the inherent MMO activity. It was felt that the only way in which this could be made into a continuous process was to develop ways of reactivating inactivated cells. This will be considered in the next Chapter.

CHAPTER 6

The Reactivation of Cells which have been Inactivated by Acetylene or Propylene Oxide

6.1 Introduction

Because of the unexpected result of inactivation following the production of PO, a method must be devised to obviate the sensitivity of the biocatalyst. Under the conditions of high PO production, the cells can be used for a maximum of 30 minutes. It is obvious therefore that this short life is inadequate if this process is to be of commercial use. One possible approach to this is the extraction of the product during the biotransformation process. Miyawaki et al. (1986) applied the solvent extraction technique to Nocardia corallina (B276) in the PO production process, but no significant improvement was obtained. Furuhashi et al. (1986) succeeded in accumulating and commercializing the R-form of long-chain epoxides by the process of adding on hexadecane as the solvent to the reaction mixture. Using the fed batch reaction method, the concentration of 1,2-epoxytetradecane in the reaction mixture reached 80 g/l within 6 days (Furuhashi and Takagi, 1984). If a high specific productivity is not essential in such a production of chiral epoxide, and if the main reason for inactivation is the accumulation of the product, then a two phase system is sometimes effective for the production of fine chemicals.

In order to reduce the concentration of PO in the reaction mixture of Methylococcus capsulatus (Bath), many types of solvents: liquid alkanes and alkenes, C_4 - C_8 alcohol and ketones, ethers, esters, nitriles, heterocyclic compounds and vegetable oils were tested. However, Methylococcus capsulatus (Bath) was quite unstable under the conditions of water-solvent mixture. Most of the solvents inactivated cells within 30 minutes. Perfluoro-1-octanol and vegetable oils such as corn oil, sesame oil, did not inactivate the cells, however only 60 - 70% of PO was extracted to the solvent layer when water solvent ratio was 1:1 (v/v). This extractive efficiency was not satisfactory.

Before one can attempt continuous removal of PO from the reaction mixture one must first find a means of prolonging the half-life of the short-lived cells for the development the PO production process. To overcome the short-life of the biocatalyst, a programme aimed at the isolation of a PO-tolerant strain was initiated.

Epoxides are reactive electrophiles and it has been clearly established that some of them bind to macromolecular compounds (Lawley and Jarman, 1972) and exert a mutagenic (Huberman et al., 1971; Amas et al., 1972) and carcinogenic effect (Maqurd et al., 1972). Therefore PO was used as a mutagen. The possibility of isolating a PO-tolerant strain is dependent on how many proteins or enzymes within the cells are sensitive to the epoxide. PO was added to the 10 L chemostat culture of Methylococcus capsulatus (Bath) at one time or continuously at a concentration of about 1mM, however no PO-tolerant strain was found. Accordingly, next strategy "reactivation of inactivated cells" was undertaken.

6.2 The Inactivation of MMO using Acetylene and its Reactivation in vivo

6.2.1 Introduction

The reactivation of cells which were inactivated by external PO was found throughout the process of selecting a PO-tolerant strain. The inactivation and reactivation mechanisms following the production of PO from propylene seems to be complicated because many of the effects that PO has on the alkylation of biological materials (see Tamaratani, 1986). In order to elucidate the reactivation mechanism in methanotrophs, it was first necessary to devise a reproducible inactivation procedure. Acetylene was chosen as an inactivator, since its inactivation mechanism

on MMO had already been well researched (Prior and Dalton, 1985b). The cells of Methylococcus capsulatus (Bath) or Methylocystis parvus (OBBP) were inactivated using acetylene. Acetylene was added to the cell suspensions and at the same time the methanol was supplied at a rate of 80-350 nmol/min/mg cells for 10 minutes to inactivate cells. After 10 minutes 20% methane in air was continuously supplied at a rate of 0.5 VVM throughout the experiment. The cells inactivated by acetylene were reactivated quickly and they completely restored their propylene-oxidising activity within a few hours.

With PO, inactivation required the presence of an electron donor. When acetylene was added to the reaction mixture, whole cell activity also decreased sharply in the presence of methanol as an electron donor. However, if the treatment time by acetylene was sufficiently long, the cell was inactivated without the addition of an electron donor, presumably due to the presence of endogenous energy reserves. It was possible to obtain various levels of inactivation of the cells by controlling the amount of acetylene present, the treatment time with acetylene, oxygen supply or the amounts of electron donor. Routinely, acetylene and methanol were added simultaneously and acetylene was removed by flushing the vessel with air. A period of 10 minutes was found to be sufficient to remove the 1.0 ml of acetylene from the liquid and gaseous phases of the reactor when the air flow rate was greater than 0.5 VVM.

6.2.2 The Effect of Carbon Sources in the Reactivation Process

6.2.2.1 Methane

It has been shown earlier that cells require carbon and energy source for reactivation. Fig. 6.2.1 shows the inactivation of cells (MMO) by acetylene and subsequent reactivation. No significant reactivation was observed when a carbon and energy source were absent. However, by supplying 20% methane in air and allowing three and a half hours to elapse, the cells were completely reactivated to the same level of activity which existed before acetylene was added. It was also observed that there was no cell growth during reactivation. The same effects were observed for other electron donors, namely methanol. (Fig. 6.2.2).

One question remained unanswered: if the residual activity after acetylene treatment was zero, were cells capable of reactivating when methane was used as a carbon source? In order to investigate this phenomenon, cells were inactivated by controlling the addition of acetylene. Even though MMO was entirely inactivated the cells could be completely reactivated. The results are shown in Fig. 6.2.3. This phenomenon can be understood if one bears in mind that the cells have an endogenous carbon and energy source which allows reactivation of MMO to a minimal level of activity, so making oxidation of methane possible. This oxidation of methane inturn provides an energy source for the further oxidation by MMO. The involvement of an endogenous energy source in reactivation of MMO was shown by using Methylocystis parvus (OBBP) as an example. The cells, which had been completely inactivated by acetylene, were reactivated without any external carbon and energy source, but this reactivation was found to be only 50% of the original activity (Fig. 6.2.4). The same cells were

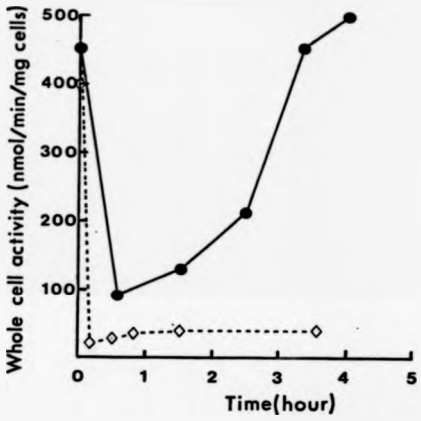


Fig. 6.2.1 The effect of methane as a carbon source in the reactivation process

◇ No carbon source

● methane as a carbon source

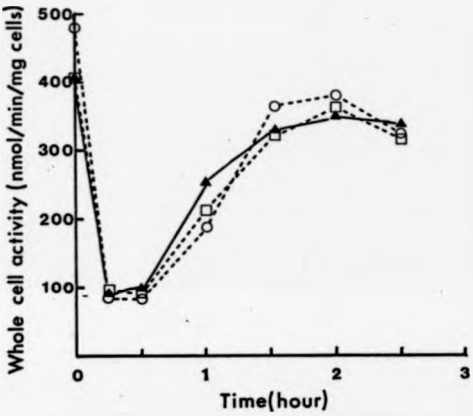


Fig. 6.2.2 The effect of methanol as a carbon source in the reactivation process

Electron donor ○ 1mM Methanol
▲ 2mM Formaldehyde
□ 100mM Formate

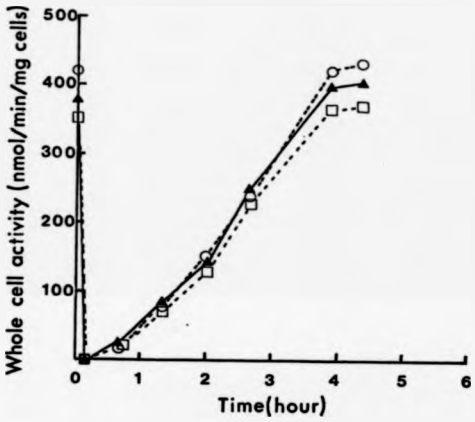


Fig. 6.2.3 The reactivation of Methylococcus capsulatus (Bath) cells in which MMO was completely inactivated by acetylene

Electron donor ○ 1mM Methanol
▲ 2mM Formaldehyde
□ 100mM Formate

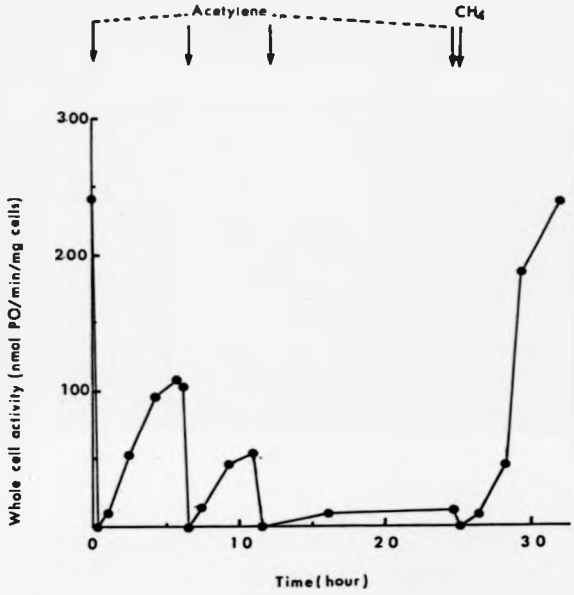


Fig. 6.2.4 The reactivation of acetylene-inactivated cells by the endogenous energy in *Methylocystis parvus* (OBBP)

further subjected to inactivation by the pulsed addition of acetylene, again reactivation occurred but this time only 20% of the initial activity was restored. No more reactivation was observed following the third period of inactivation. Methylocystis parvus (OBBP) is known to accumulate poly-beta-hydroxybutyrate in the cell (Asenjo and Suk, 1986), and this may well be used as an energy source for reactivation. An accumulation of acetic acid in the cell suspension (1.7mM) was observed 12 hours after the first inactivation. When methane was supplied after the fourth inactivation treatment, a rapid reactivation of cell activity occurred reaching the same level of activity as before observed with the first inactivation. From this series of investigations, it was found that a carbon source, either external or internal, was necessary for reactivation.

The concentration of methane in air was found to have an effect on the reactivation (Fig. 6.2.5). A depression of reactivation was observed during the first 2 hours when 3-9% of the methane supplied was relieved by increasing the methane concentration to 16%. At about 20%, maximum reactivation rate was obtained.

The depression in the early stages of the reactivation process, when using low concentrations of methane, appears to be responsible for lowered methane consumption, therefore reactivation of cell activity (MMO) must be effected by biomass concentration and gas flow rate as well as gas composition.

6.2.2.2 Methanol

Further investigations were carried out using methanol as a possible source of carbon and energy for the reactivation of MMO. After inactivation of MMO by acetylene, methanol was added continuously at a rate of 330 nmol/min/mg cells to the culture. MMO activity recovered on addition of methanol. The reactivation rate of the cells when supplied

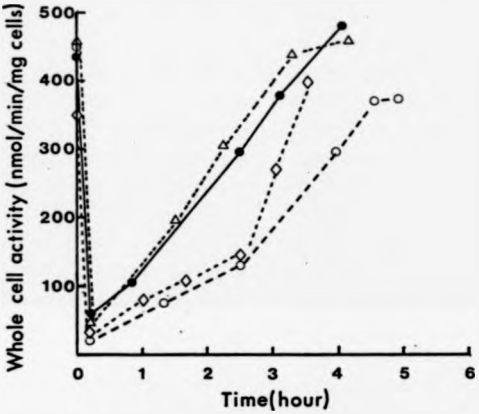


Fig. 6.2.5 The effect of methane concentration in air during the reactivation process in Methylococcus capsulatus (Bath)

Methane concentration
(%)

○ 3
◇ 9
△ 16.5
● 23

with methanol appeared to be 15% greater than with methane (Fig. 6.2.6). This might be caused by the greater efficiency of methanol as an energy source.

6.2.2.3 Formaldehyde

The novel result was obtained which demonstrated that formaldehyde was effective as a carbon and energy source in the reactivation of MMO (Fig. 6.2.7). When formaldehyde was supplied continuously at the rate of 50 or 100 nmol/min/mg cells, cells were reactivated but after 150 minutes, the reactivation ceased followed by a gradual loss of activity. Formaldehyde is an important intermediate for the assimilatory and dissimilatory pathways. Reed (1976) demonstrated that the formaldehyde could be utilised as the sole carbon and energy in Methylococcus capsulatus (Bath). Since that report, there have been no observations on the use of formaldehyde as a carbon and energy source for cell growth. Eccleston and Kelly (1973) reported that formaldehyde was not utilised as the carbon source for cell growth but [^{14}C]-labelled formaldehyde was found to be converted into amino acids. Where the reactivation of MMO is concerned, it appears that formaldehyde is used as the carbon and the energy source and probably the carbon source for amino acid and protein synthesis. Incomplete reactivation which occurs during utilization of formaldehyde can be considered to be due to the toxic and inactivatory effect of formaldehyde throughout the reactivation. Effects of formaldehyde on the reactivation will be discussed further in Chapter 8.

6.2.2.4 Formate

No reactivation was observed when formate was supplied continuously at a rate of 30 nmol/min/mg cells (Fig. 6.2.8). Formate is oxidised by

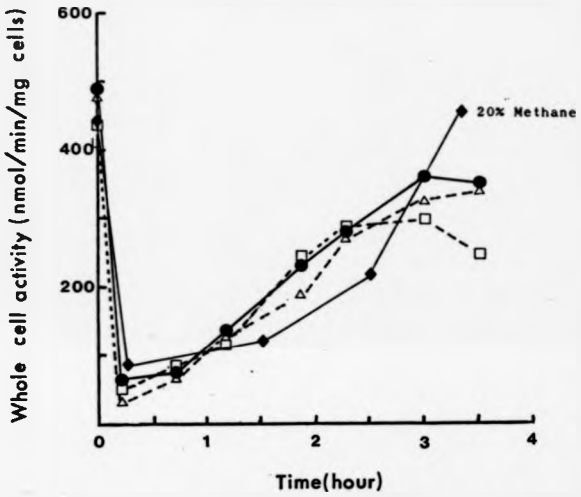


Fig. 6.2.6 The affect of methanol on the reactivation rate of the inactivated cells in Methylococcus capsulatus (Bath)

Electron donor ● 1mM Methanol
△ 2mM Formaldehyde
□ 100mM Formate

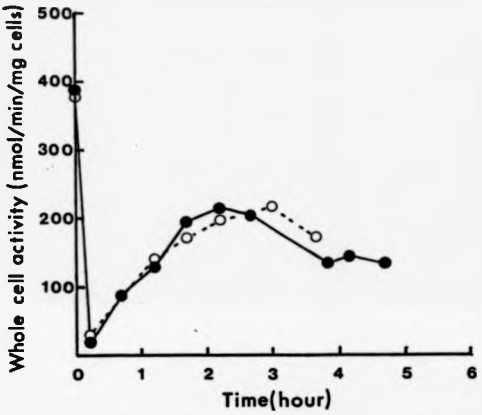


Fig. 6.2.7 The effect of formaldehyde on the reactivation as a carbon and energy source in Methylococcus capsulatus (Bath)

Formaldehyde addition rate (nmol/min/mg cells)

○ 50
● 100

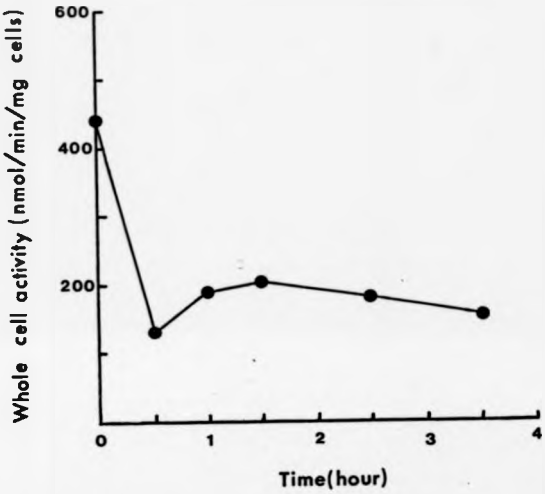


Fig. 6.2.8 The effect of formate on the reactivation as a carbon and energy source in Methylococcus capsulatus (Bath)

formate dehydrogenase, thus providing NADH and carbon dioxide. This result is consistent with the requirement of a source of carbon for the reactivation of MMO and not with just a source of energy.

6.3 The Effect of Oxygen on the Reactivation of MMO

Methane is used as a carbon and energy source in process of reactivation. Since the oxidation of methane is necessary before it can be used as a carbon source, then oxygen also must be necessary for reactivation. The oxygen in the gase phase was replaced by methane or 20% methane in nitrogen shown in Fig. 6.3.1. No reactivation was observed when acetylene-inactivated cell suspensions were incubated without oxygen. A hundred and ten minutes later, 20% methane in air was supplied to the cell suspensions which stimulated the reactivation of MMO.

6.4 The Effect of Nitrogen Sources on the Reactivation

Methylococcus capsulatus (Bath) can use both inorganic and organic sources of nitrogen for growth, such as nitrate, dinitrogen, ammonia, glutamine or asparagine (Murrell, 1981). An experiment was undertaken to determine whether the nitrogen source is necessary or not for the reactivation of MMO. Acetylene-inactivated cells of Methylococcus capsulatus (Bath) and Methylocystis parvus (OBBP) were washed three times with potassium nitrate-free NMS medium. A slow reactivation was observed when dinitrogen was supplied to Methylococcus capsulatus (Bath) but this reactivation rate was also observed when dinitrogen was replaced with argon (Fig 6.4.1). This suggested that endogenous nitrogen was being used providing a slow reactivation. On the contrary, when 0.5 g/l potassium nitrate was added, cells were reactivated and its reactivation rate was 3 times faster than that of the endogenous

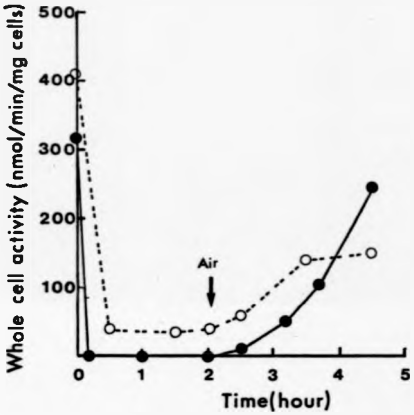


Fig. 6.3.1 The effect of oxygen on the reactivation of MMO in Methylococcus capsulatus (Bath)

100% methane (○) or 20% methane in nitrogen (●) was supplied for 110 minutes, then 20% methane in air were supplied.

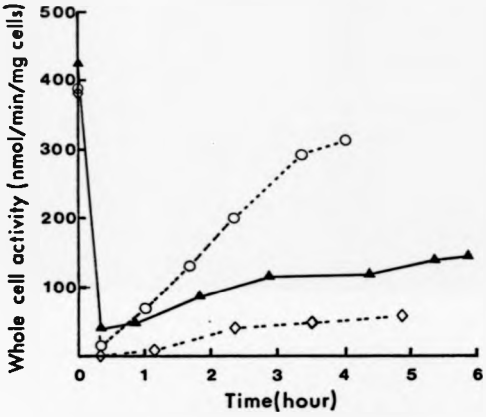


Fig. 6.4.1 The effect of nitrogen sources on the reactivation of MMO in Methylococcus capsulatus (Bath)

Nitrogen source

KNO_3, 0.5 g/l(○), dinitrogen(Air, ▲), no nitrogen source(20% O_2 in argon, ◇)

nitrogen source.

On the other hand, acetylene-inactivated cells of Methylocystis parvus (OBBP) were reactivated when dinitrogen was supplied (Fig. 6.4.2). The reactivation rate with dinitrogen or endogenuous nitrogen source as a nitrogen source was about 60% of that of nitrate as a nitrogen source. Probably Methylocystis parvus (OBBP) used the endogenous nitrogen source in addition to the endogenous carbon source. In this case it is clear that both the nitrogen source and carbon source are necessary for reactivation.

6.5 The Effect of Sulphur Sources

Sulphur plays an important role in monooxygenases due to the presence of metal-sulphur cluster. However, the effect of the sources of sulphur on cell growth or enzyme activity have not been studied in detail in methane-oxidising bacteria. Usually magnesium sulphate is used as a source of sulphur. It was assumed that if MMO was newly-synthesized during the reactivation process, sulphur must be required. Methylococcus capsulatus (Bath) was used for these experiments.

Cells inactivated by acetylene required a sulphur source for the reactivation of MMO. No reactivation was observed when sulphur was omitted from medium (Fig. 6.5.1). Not only sulphate but also hydrogen sulphide and methionine were effective sources of sulphur. No reactivation was obtained when cysteine was supplied (Fig. 6.5.2). The failure of cysteine to effect reactivation could have been caused by the oxidation of cysteine to cystine during the reactivation process. Cysteine was oxidised when it was added to the cell suspensions, and lots of precipitate (cystine) were formed. Cells also may be unable to be utilise it because of its low solubility.

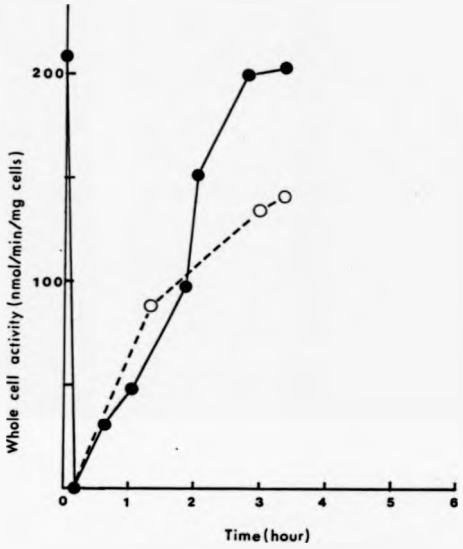


Fig. 6.4.2 The effect of the nitrogen sources on the reactivation of MMO in Methylocystis parvus (OBBP)

Nitrogen source

KNO_3 ,1.0 g/l(●),dinitrogen(Air, ○)

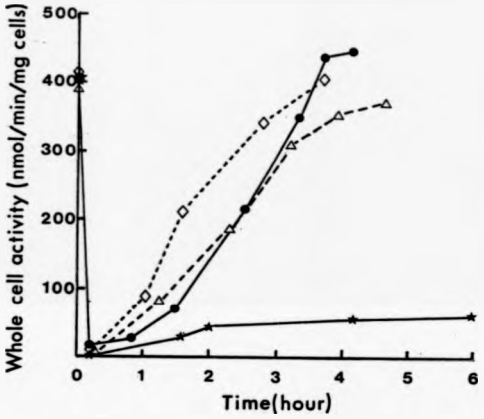


Fig. 6.5.1 The effect of various sulphur sources on the reactivation of MMO in Methylococcus capsulatus (Bath)

Sulphur source

- ● Na_2SO_3 500 mg/l
- △ Methionine 25 mg/l
- ◇ NaSH 50 mg/l
- * No sulphur source

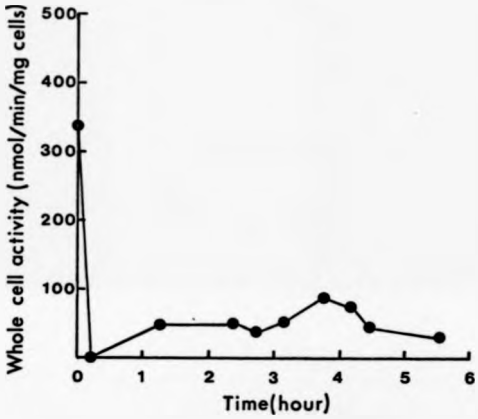


Fig. 6.5.2 The effect of cysteine as a sulphur source on the reactivation of MMO in Methylococcus capsulatus (Bath)

6.6 The Effect of Temperature on the Reactivation

Methylococcus capsulatus (Bath) can grow in temperatures ranging from 34°C to 47°C. An experiment involving inactivation was undertaken to determine the effect of temperature on the reactivation of MMO. The results are shown in Fig. 6.6.1. Temperature was also found to be important for the reactivation of MMO. The maximum reactivation rate was obtained at a temperature of 45°C. It was also noted that the reactivation rate slowed down by decreasing the temperature. No reactivation was observed in two particular areas, below 32.5°C and above 48°C. This experiment also revealed that some of the enzymes associated with the assimilation pathway were affected by temperatures below 32.5°C and above 48°C. The enzymes of dissimilation pathway were active because propylene was oxidised at a temperature below 32.5°C and above 48°C when methanol was used as an electron donor.

6.7 The Reactivation of Cells which are Inactivated by Extracellular PO

By way of preparation for the investigation on the reactivation of cells which were inactivated under the conditions of PO production (propylene-inactivated cells), it was necessary to make a comparison in reactivation between cells which were inactivated by acetylene (acetylene-inactivated cells) and cells which were inactivated by externally added PO or externally accumulated PO (PO-inactivated cells). PO which was added exogenously to the cell suspensions was found to act as an inactivator of MMO and formate dehydrogenase *in vivo* using extracted enzymes analysis by Stanley and Richards (unpublished data). The inactivation mechanism of PO-inactivated cell appeared simpler than the propylene-inactivated cells. Accordingly, the reactivation pattern of PO-inactivated cells was investigated.

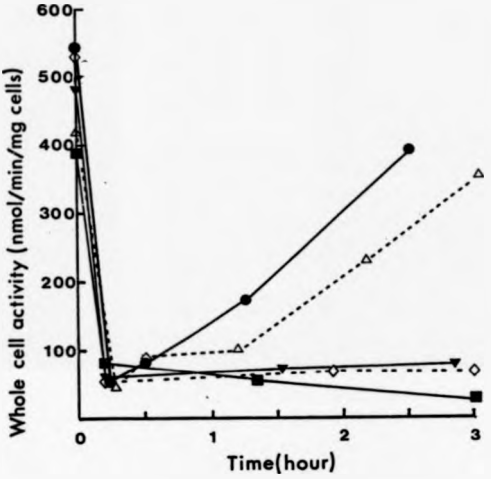


Fig. 6.6.1 The effect of temperature on the reactivation of MMO in Methylococcus capsulatus (Bath)

Reactivation temperature (°C)

■ 45

● 45

△ 37.5

▼ 32.5

◇ 4

6.7.1 Experimental

A similar method of that of the previous experiment, that is acetylene-inactivated cells, was used to study the reactivation of PO-inactivated cells. PO was added to the cell suspensions and at the same time the methanol supply was started at a rate of 330 nmol/min/mg cells for 30 minutes. Then the methanol supply was cut off and air was supplied at approximately 20 VVM for 15 to 20 minutes in order to scrub out the PO. When the PO concentration decreased to the level of up to 0.03mM, the supply of air was cut off and 20% of methane in air was supplied at the rate of 0.5 VVM. Cell activity was analysed at irregular intervals. Methylococcus capsulatus (Bath) was used throughout the experiments.

6.7.2 Results and Discussion

The results are shown in Fig. 6.7.1a. Cells lost activity as a result of the pulsed addition of 8mM PO so that after 30 minutes, they retained only 5% of their initial activity. However, whole cell activity reactivated quickly. It was considered that if the inactivation mechanisms were totally different between acetylene and PO, then their reactivation patterns might be different. Therefore, cells were inactivated initially by acetylene and then treated with 8mM PO for 30 minutes. The reactivation pattern of cells after scrubbing out the acetylene and the PO was very similar to that of only 8mM PO-treated cells (Fig. 6.7.1b). This suggests that acetylene and PO inactivate cells in a similar manner. No significant difference was observed in rate of reactivation between acetylene-inactivated cells and the cells inactivated by concentrations of PO varying between 1-8mM (Fig. 6.7.1, 6.7.2). The reactivation of whole cell MMO activity measured using 1mM methanol, 2mM formaldehyde and 100mM formate as electron donors revealed

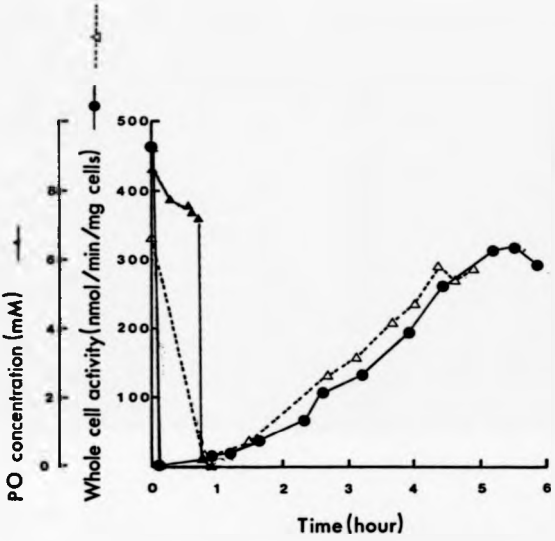


Fig. 6.7.1 The reactivation of the PO-inactivated cells and both PO- and acetylene-inactivated cells of Methylococcus capsulatus (Bath)

a △ PO-inactivated cells

b ● PO- and acetylene-inactivated cells

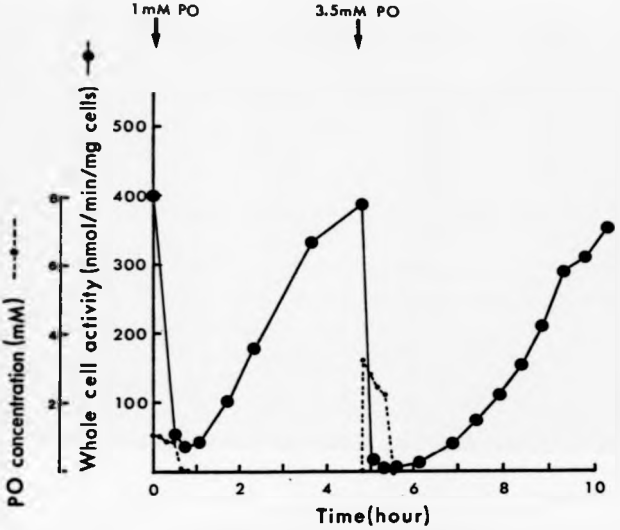


Fig. 6.7.2 The effect of external PO concentration on the inactivation and the reactivation of cells of Methylococcus capsulatus (Bath)

similar patterns for each different electron donor (Fig. 6.7.3). This also suggests that the reactivation of whole cells which have been inactivated by external PO is due to the reactivation of MMO. The similarity of reactivation pattern in acetylene-inactivated cells and PO-inactivated cells suggests that PO is a potent inactivator in a similar way to acetylene.

6.8 Repeated Reactivation of Cells Following Inactivation

It has been shown that cells inactivated by either acetylene or external PO can be reactivated to regain the same level of activity after inactivation as before inactivation. However, the question arises as to how many times cells can be reactivated completely? If this reactivation mechanism is to be applied to the continuous PO production process, then repeated reactivation must occur. Repeated reactivation was tested by the multiple addition of either acetylene or PO to the cell suspensions.

The result is shown in Fig. 6.8.1. Methylococcus capsulatus (Bath) was used for this experiment. It indicated that the inactivation/reactivation cycle was capable of being repeated many times, and that the cells were reactivated almost completely on each occasion. No decrease in reactivation rate was observed in such an experiment.

6.9 Protein Synthesis during the Reactivation

The reactivation condition which have been investigated suggests that reactivation is accompanied by protein synthesis. To confirm this assumption, chloramphenicol, an inhibitor of protein synthesis and rifampicin, an inhibitor of RNA polymerase were used to determine whether reactivation was inhibited or not by these two inhibitors.

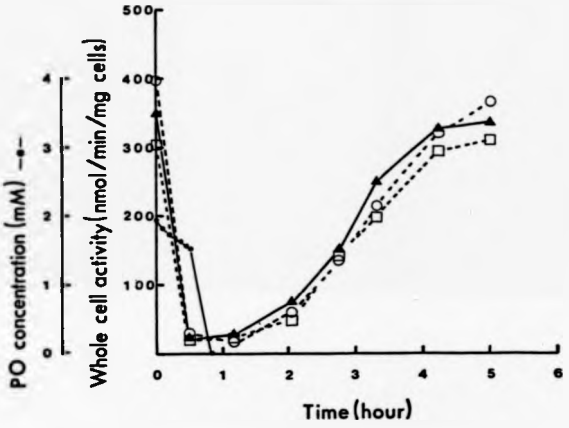


Fig. 6.7.3 The reactivation of whole cell MMO activity of the PO-inactivated cells measured using different electron donors

○ 1mM methanol

▲ 2mM formaldehyde

□ 100mM formate

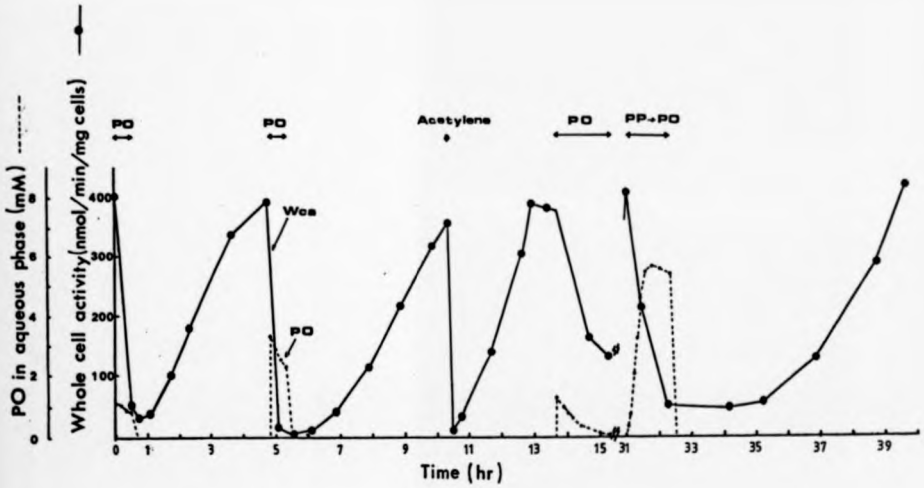


Fig. 6.8.1 Repeated reactivation of cells following inactivation by acetylene and externally added PO

The treatment concentrations were decided from the minimum inhibition concentration for cell growth. Methylococcus capsulatus (Bath) and Methylocystis parvus (OBBP) were used for this experiment. A concentration of 150 μg/ml for chloramphenicol and 2.5 μg/ml for rifampicin were used.

The cells were inactivated by acetylene and then cell suspensions were subdivided into 250ml flasks. Three flasks which contained 20ml of cell suspension were prepared for both strains. Chloramphenicol or rifampicin was added to each flask (plus a control with no addition) and sealed with a Suba-Seal stopper, then 50ml of methane was added after removal of an equal amount of air and the flasks were incubated at 45°C for Methylococcus capsulatus (Bath) and 30°C for Methylocystis parvus (OBBP). Reactivation of whole cell activity was monitored and the results are shown in Fig. 6.9.1a and Fig. 6.9.1.b.

In Methylococcus capsulatus (Bath), reactivation was completely inhibited by the addition of chloramphenicol. However cells were partially reactivated if any rifampicin was present. In Methylocystis parvus (OBBP), chloramphenicol inhibited the reactivation strongly, but rifampicin did not. Strong inhibition of reactivation by chloramphenicol suggested that protein was synthesized during the reactivation process. Chloramphenicol and rifampicin inactivate the whole cell activity (Wca) itself. When the cells of Methylocystis parvus (OBBP) were treated with the same amount of chloramphenicol or rifampicin, whole cell activities were decreased to 60% of the initial activity within 4 hours (see Fig. 6.9.1b). Therefore, antibiotics themselves accelerate inactivation.

6.10 Discussion

The work presented in this Chapter provides the first report of reactivation of MMO following inactivation by acetylene. In 1984, Hou

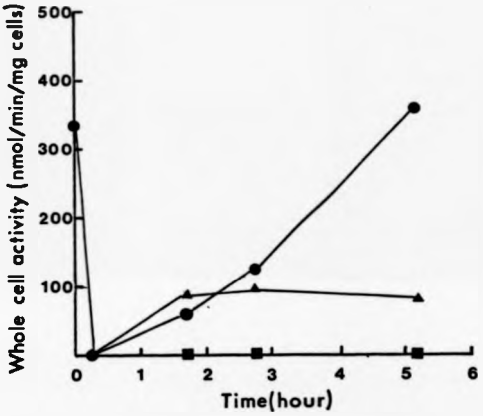


Fig. 6.9.1a Protein synthesis during the reactivation of MMO in Methylococcus capsulatus (Bath)

■ Chloramphenicol 150 µg/ml

▲ Rifampicin 2.5 µg/ml

● Control

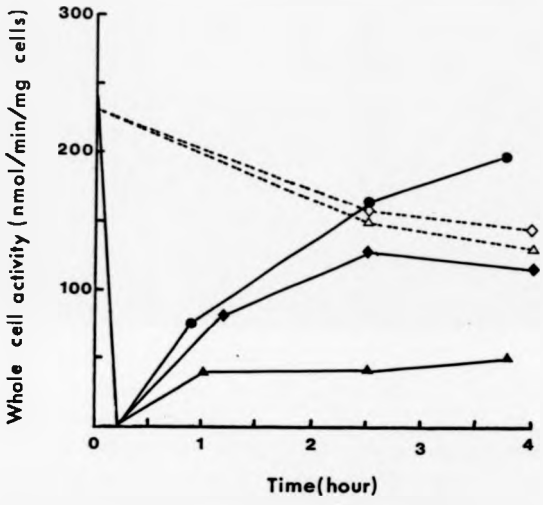


Fig. 6.9.1b Protein synthesis during the reactivation of MMO in Methylocystis parvus (OBBP)

Normal cells and acetylene-inactivated cells were treated with chloramphnicol (△, ▲) and rifampicin(◇ , ◆).
● Control

demonstrated a regeneration of PO production system using a new type of gas-solid bioreactor in which methanotroph, Methylosinus sp. CRL 31 was packed (Hou, 1984b). The cell paste was coated on porous glass beads then the glass beads were packed in a small glass reactor. A mixture of propylene and oxygen (1:1, v/v) was introduced, however no electron donor was supplied externally. PO was produced over a period of 12 hours at a rate of
18 μ moles/h/20mg protein (15 nmoles/min/mg protein) for the first 7 hours. However after 12 hours of operation, PO production stopped. In situ regeneration of the biocatalyst in the bioreactor was conducted after 12 hours of operation using methanol as the regeneration substrate. By this regeneration, the production of PO immediately resumed at a constant rate of 12 μmol/hr.

The regeneration of PO production which Hou (1984b) investigated appears not due to a reactivation of inactivated cells, because as he had noted, the cessation of PO production was due to the depletion of endogenous energy. Accordingly, regeneration of PO production was initiated by the supply of energy (methanol) but appeared not to be responsible to the reactivation of inactivated cells.

First report on the reactivation of oxygenase activity which had been inactivated during the immobilization of cells was demonstrated by Somerville et al. (1977) in benzene-oxidising bacteria, Pseudomonas putida. They noted that when cells were immobilised in polyacrylamide gel, cells lost 40-70% of their activity, however this activity was restored by incubation in a medium containing benzene and succinate. They also found that partial reactivation could be achieved by incubation with iron salt, in the absence of a carbon source. They suggested that the reactivation was the co-operative action of reactivation of enzymes and cell-growth. In 1985, Habets-Crützen and de Bont demonstrated that the cells of ethylene-utilising bacteria, Mycobacterium E3 which had been inactivated by the addition of 50 mM PO

could be reactivated. When PO-inactivated cells were incubated using ethylene as a carbon source, cells were reactivated, however it was not clear whether the rectivation of whole cell activity was responsible for the reactivation of cells per se or whether fresh growth of the cells was the major factor. In their experiment, the activity of control (no PO treated cells) was also increased.

In 1987, Suzuki and co-workers observed that the thermophilic methane-oxidising bacterium Methylococcus sp. (MN-222) which was completely inactivated during storage at 45°C was reactivated by the addition of 10mM methanol. When methanol was added, the cells consumed methanol and whole cell MMO activity was completely reactivated within two hours. However, the cells which were inactivated either by exogenous PO or under the conditions of PO production were not reactivated. It can be interpretated the reason why Methylococcus sp. (MN-222) was not reactivated was due to the lack of nutrients such as nitrogen source or sulphur source. That experiment was carried out using 20 mM phosphate buffer. It is considered the mechanism of reactivation of cells which are inactivated during the storage is different compared to the reactivation of acetylene- or PO-inactivated cells. Reactivation of Methylococcus sp. (MN-222) did not appear to be associated with protein synthesis. The inactivation of Methylococcus sp. (MN-222) during the storage may be due to the conformational change and in this case a small amount of energy (methanol) might be enough to reconstitute the active structure.

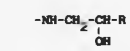
It was found that the carbon source, nitrogen source, sulphur source, temperature and oxygen were all necessary for the reactivation of acetylene- or PO-inactivated cells. Probably other elements such as phosphate, potassium, magnesium, calcium or trace elements are also required for repetition of reactivation especially when cell growth is

associated with the reactivation. Reactivation and cell growth are basically independent because cells can be reactivated without any cell growth. The relation between reactivation and cell growth will be discussed in Chapter 10.

Although reactivation is independent of cell growth it is certainly dependent on protein synthesis. It is easy to assume that the protein synthesis is associated with the reactivation process by the reactivation conditions which mentioned above and by the inhibition of chloramphenicol. Tawaratani et al. (1980) demonstrated that the germination of spores of Bacillus subtilis which were treated with PO was also inhibited by chloramphenicol. In the above case it was not known which enzymes or part of the cell materials were reactivated. However, in the case of acetylene-inactivated cells, it is obvious that it is MMO which is inactivated and reactivated. No report concerning the reactivation of an enzyme which is inactivated has been demonstrated in methanotrophs.

It has been discussed that the PO externally added to the cell suspension must inactivate MMO and that the inactivation process was considered to be similar to that by acetylene. The reactivation patterns of acetylene-inactivated cells and PO-inactivated cells closely resembled each other. These similarities in reactivation patterns also supports that the external PO inactivates the MMO active centre. However, the alkylation mechanisms of acetylene (ketene) and PO (PO itself or unknown intermediate) might be different. Kunze et al.. (1983) reported that the alkylation of the porphyrin ring of hepatic microsomal cytochrome P-450 by olefins and acetylenes were different and that olefins alkylated the nitrogen of pyrrole ring (C or D) but acetylenes alkylated that of pyrrole ring A. They also showed the alkylated form of porphyrin by olefins and acetylenes. These differences are shown below:

a) Olefins



b) Acetylenes

From their results it can be considered that the intermediates of alkylation by olefins are epoxides but the intermediates of acetylenes are allene oxides such as ketene which has been postulated in Methyloccus capsulatus (Bath) by Prior and Dalton (1985b). However the epoxides must be activated by the oxygenase before it alkylates the active centre as discussed in Chapter 4. Alkylation positions in MMO by acetylene (ketene) and PO may differ slightly in methanotroph. However reactivation patterns in both cases are similar suggest the reactivation mechanism may well be the same.

Methane monoxygenase is essential to methanotrophs. However, this enzyme is not very stable, since under co-oxidative conditions MMO readily loses its activity. In order to survive in the biosphere, this reactivation system may play an important role for these organisms. For example, after a long resting period when the cell starts to grow again they can reactivate MMO which has been inactivated during the resting conditions. Whether the reactivation mechanism is controlled or not is an interesting point. This subject is further discussed in Chapter 9.

One other interesting point is the repetition of reactivation which is essential for the PO production process. However in this Chapter inactivation by external inhibitor (PO or acetylene) has been considered whereas in a process PO would be continuously produced during biotransformation. Therefore the reactivation of cells under conditions of in vivo PO production will now be considered.

In the next Chapter, the main subject on reactivation: the

reactivation of cells which are inactivated under the conditions of PO production will be demonstrated.

CHAPTER 7

Inactivation of Cells under the Conditions of PO Production and its Reactivation

7.1 Introduction

The studies on the inactivation of cells under conditions of PO production, using the immobilized cell-biofilm reactor as discussed in Chapter 5 suggested that the inactivation of cells caused by PO accumulation in the reaction mixture and inactivation following the oxidation of propylene were independent of each other. It was also evident that PO inactivated MMO, but it is not yet clear why cells are inactivated without the accumulation of external PO. If the inactivation mechanisms of cells following the production of PO are similar to that of PO-inactivated cells from externally added PO, then the reactivation processes must resemble each other. In this Chapter, the reactivation of cells which are inactivated under the conditions of PO production is demonstrated. If propylene-inactivated cells can be reactivated in a similar manner to PO-inactivated cells, the potential for the development of PO production process will be increased.

7.2 The Reactivation of Cells Inactivated under the Conditions of PO Production

When propylene was fed to the fermenter, PO was accumulated quickly in the reaction mixture and at the same time, cells lost their activity. The peak productivity in this experiment was calculated from the accumulation rate of PO in the reaction mixture. When the reaction was started, PO concentration in the reaction mixture was analyzed at 3 or 4 minute intervals. The highest accumulation rate was usually obtained within 10 minutes after the reaction was started and was designated as peak productivity.

Results from the IBF-reactor indicated that the increase of peak productivity accelerated the inactivation and consequently reactivation

appeared to be delayed. In order to study the relationship between peak productivity of PO on inactivation and reactivation, the following factors were varied:- electron donor, biomass and dissolved oxygen in the fermenter. Methane was used as a carbon source for the reactivation and Methylococcus capsulatus (Bath) was used throughout the experiments in this Section. When peak productivity of the cells was relatively low (35mU/mg cells) compared with their potential productivity (Wca: 380 mU/mg cells), the inactivation rate of cells was also low (2.2 mU/min) (Fig. 7.2.1). Even after 1.5 hours, the cells were still 50% of their initial activity. Reactivation after removal of PO was quite straightforward and cells were completely reactivated after a period of 2.5 hours. A similar result was obtained when peak productivity was 75 mU/mg cells (Fig. 7.2.2). During the reactivation process, no cell growth was observed.

When peak productivities of the cells were further increased by escaping from oxygen-limitation, cells lost their activity quicker. Fig. 7.2.3 shows the result of the reactivation of cells which were inactivated at a peak productivity of 170 mU/mg cells. The residual activity after 30 minutes reaction period was only 5% of the initial activity. An increase in peak productivity also increased the time required for complete reactivation. Furthermore, as peak productivity increased, a 'lag period' was observed. During this lag period, no reactivation could be outwardly observed. This period was observed at the beginning of the reactivation treatment.

When peak productivity was more than 200 mU/mg cells, the lag period lengthened (Fig. 7.2.4) and the reactivation rate, during recovery, was decreased. Sometimes, even after 7 hours, no reactivation was observed at all in conditions of high peak productivity (365 mU/mg cells).

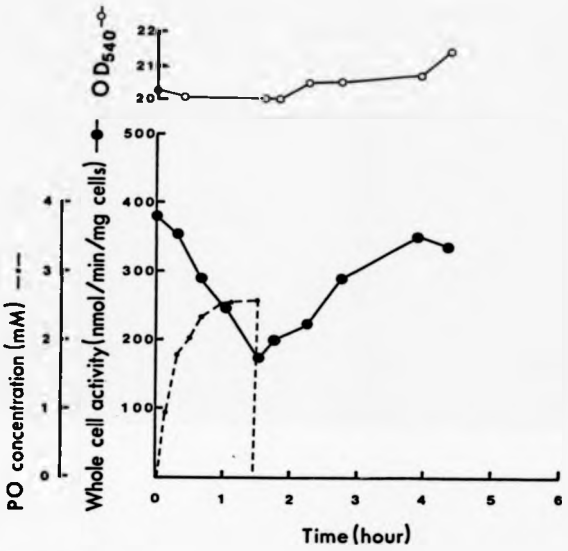


Fig. 7.2.1 The reactivation of cells inactivated under the condition of PO production - low PO productivity (I)

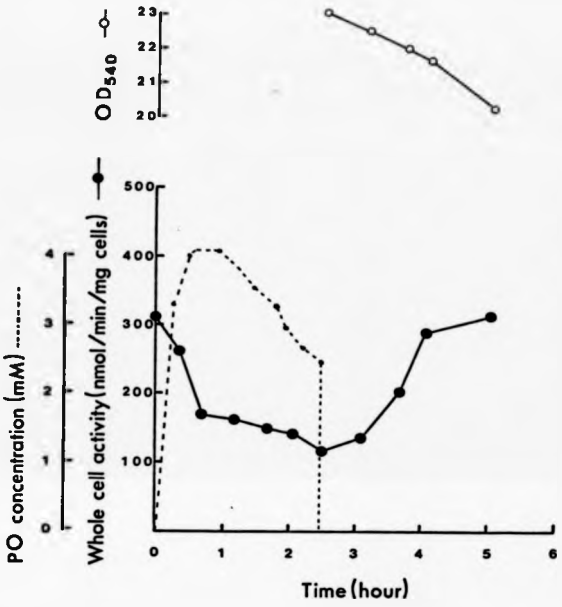


Fig. 7.2.2 The reactivation of cells inactivated under the condition of PO production - low PO productivity (II)

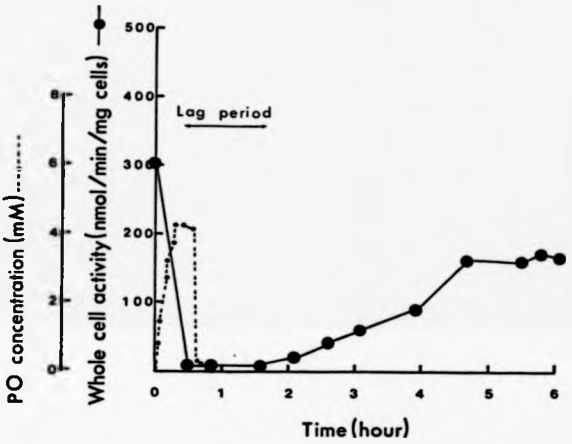


Fig. 7.2.3 The reactivation of cells inactivated under the condition of PO production - moderate productivity

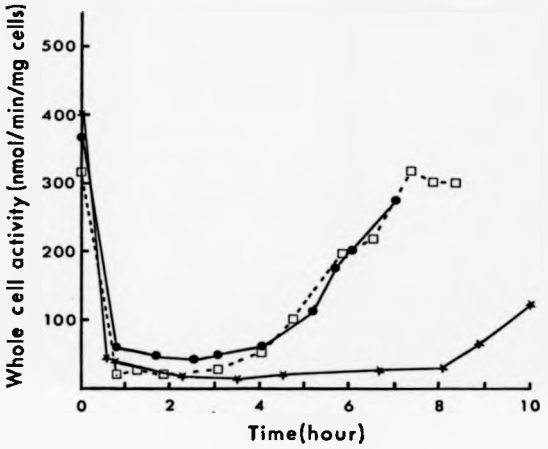


Fig. 7.2.4 The reactivation of cells inactivated under the condition of PO production — high PO productivity —

Peak Productivity (nmol/min/mg cells)

□ 205

● 250

* 365

Fig. 7.2.5 shows the relationship between residual activity at the beginning of the reactivation process and the time required for complete reactivation. When residual activity was low, it took longer for the cells to be completely reactivated. However, this phenomenon was mainly due to the prolongation of the lag period and to the low reactivation rate during the initial reactivation period.

However, the reactivation rate in the exponential phase was similar in each experiment at 1.1 - 1.5 mU/mg cells (Fig. 7.2.6) and this was not dependant on peak productivity. Peak productivity must be accelerating inactivation which is not due to the inactivation of MMO but the inactivation of some other important mechanism. Because of this 'concealed inactivation', the lag period is prolonged and initial reactivation rate is decreased. The residual activity, that is, the activity at which reactivation treatment is started, may also have an effect on the initial reactivation rate, because low Wca causes lower carbon assimilation, when methane is used as carbon source for the process of reactivation. However, if inactivation is restricted to the active site of MMO then residual activity would have little effect on the initial reactivation rate as is observed in acetylene-inactivated cells or PO-inactivated cells (see Chapter 6). Accordingly, the initial lag period in the reactivation process, seems to be due to concealed inactivation. When 'concealed reactivation', which one cannot detect as the increase in Wca, is completed, the reactivation rate which is observed to be an increase of Wca becomes normal as seen in acetylene- or PO-inactivated cells, which presumably occurs without any increase in Wca, when this is completed normal reactivation occurs. This is manifested as an increase in Wca as is found following acetylene or PO inactivation. Usually Wca was slowly restored during the initial stages of reactivation of cells even in those which were highly inactivated (Fig. 7.2.7). There is the possibility that the reactivation mechanism

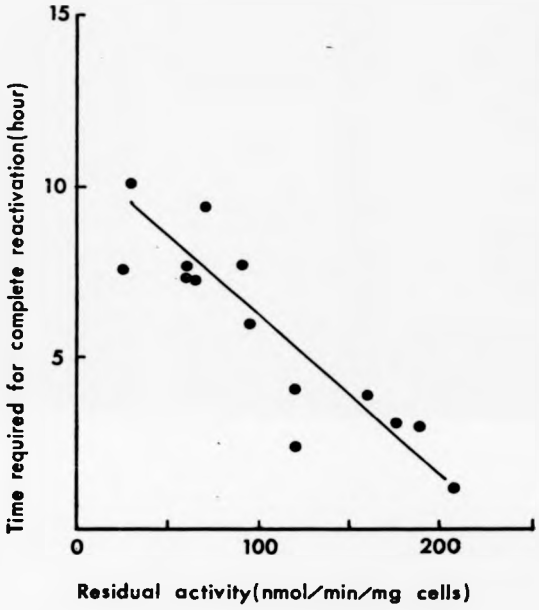


Fig. 7.2.5 The relationship between residual activity at the beginning of the reactivation process and the time required for complete reactivation

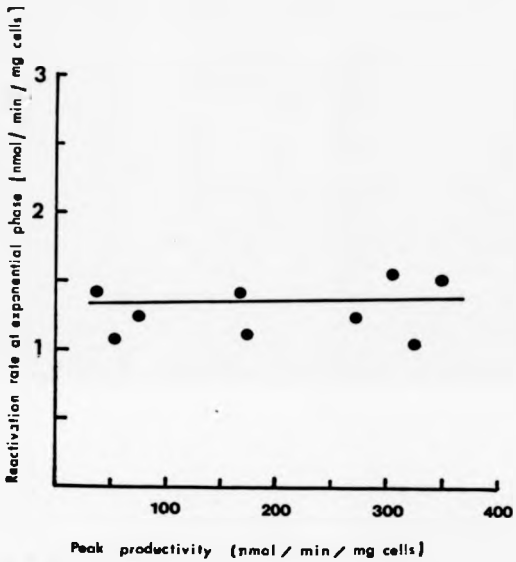


Fig. 7.2.6 The relationship between the reactivation rate in the exponetial phase and the peak productivity

for MMO is not completely destroyed, and so the repair of MMO proceeded slowly by this remaining mechanism. However, major reparation during the initial stage of reactivation appears to be concentrated on the concealed inactivation. When 'concealed reactivation' has been completed, the reactivation of MMO could then commence at maximum rate (see Fig. 7.2.7).

7.3 Effects of pH, Temperature and Carbon Sources on the Reactivation of Propylene-inactivated Cells

Due to the short life-span of the biocatalyst, the development of a PO production process would be difficult unless one uses the reactivation process. The reactivation rate affects the production costs of PO and so a high reactivation rate is necessary. Optimum reactivation conditions required for acetylene-inactivated cells have been described in the previous Chapter. However, these are the conditions required for the reactivation of particulate MMO. In case of propylene-inactivated cells however not only is MMO inactivated but some unknown system is also affected, hence the reactivation conditions may be different to the situation in which only MMO is inactivated. Furthermore, if some difference is observed between acetylene-inactivated cells and propylene-inactivated cells then this also provides information about the inactivation mechanism of propylene-inactivated cells. Effects of pH, temperature, carbon sources and copper concentration on the reactivation were therefore studied on Methylococcus capsulatus (Bath).

7.3.1 The Effect of pH on the Reactivation of Propylene-inactivated Cells

The optimum pH for the reactivation of propylene-inactivated

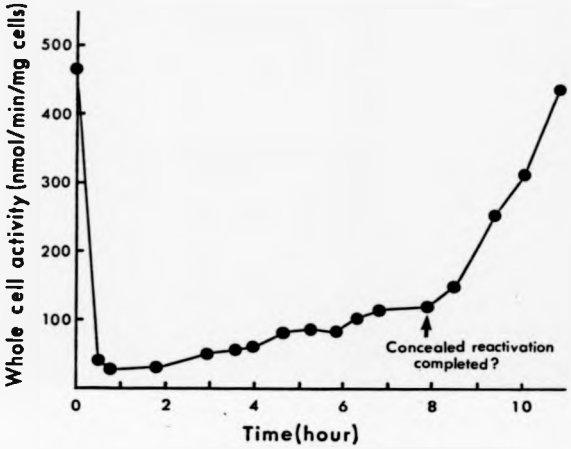


Fig. 7.2.7 The rectivation of cells inactivated under the condition of high PO production

Methylococcus capsulatus (Bath) was investigated. After removal of the PO accumulated in the reactivation following the oxidation of propylene by scrubbing, the pH was controlled with 0.5M nitric acid or 0.5M potassium hydroxide to different values. The results are shown in Fig. 7.3.1. The maximum reactivation rate was found in the range of pH 7.0 - 7.2. This optimum pH was similar or slightly higher than that of the optimum pH for cell growth in Methylococcus capsulatus (Bath) (pH 6.8).

7.3.2 The Effect of Temperature on the Reactivation of Propylene-inactivated Cells

The effect of temperature on the reactivation of cells which were inactivated under conditions of PO production is shown in Fig. 7.3.2a,b. Cells were inactivated at a temperature of 45 and 40°C. The optimum temperature for reactivation was observed to be 45°C. It was noted that no reactivation took place at temperatures lower than 30°C, likewise there was no reactivation above 48°C. The effect of temperature on the inactivation of cells appears to be similar.

7.3.3 The effect of Carbon Sources on the Reactivation of Propylene-inactivated Cells

It has already been pointed out that methanol is more effective than methane as a carbon and energy source for the purpose of reactivation, since methane has first to be oxidised by MMO (see Chapter 5). However, at an early stage of experimentation, when using methanol as a carbon and energy source for the reactivation of propylene-inactivated cells, many experiments were spoiled by the rapid decrease in pH. Following the addition of methanol, the pH was kept higher than 7.2 with potassium hydroxide, but no reactivation was

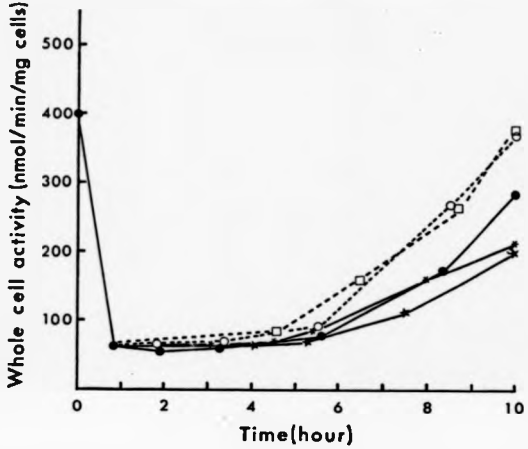


Fig. 7.3.1 The effect of pH on the reactivation of propylene-inactivated cells

Reactivation pH ● 6.5
○ 7.0
□ 7.2
✱ 7.5
★ 8.0

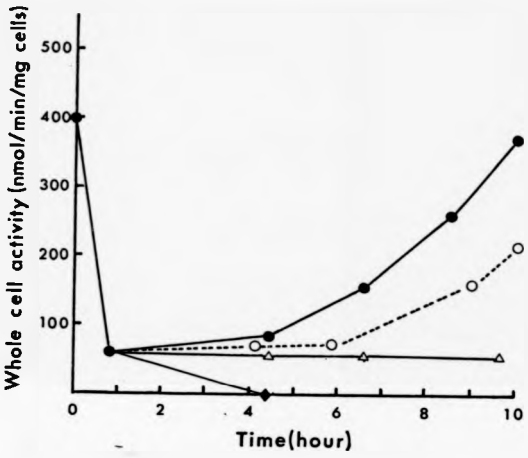


Fig. 7.3.2.a The effect of temperature on the reactivation of propylene inactivated cells of Methylococcus capsulatus (Bath). a: reaction temperature 45°C

Reactivation temperature (°C)

◆ 48

● 45

○ 37

△ 30

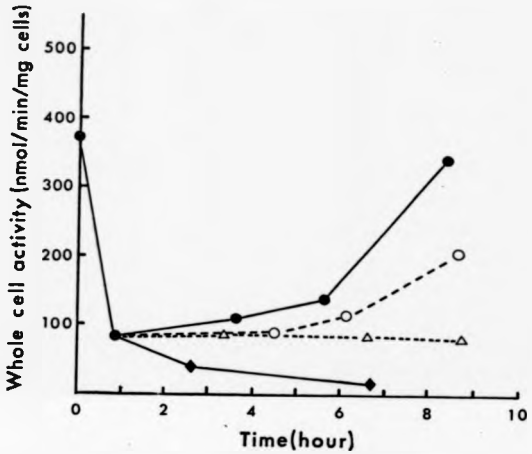


Fig. 7.3.2.b The effect of temperature on the reactivation of propylene inactivated cells of Methylococcus capsulatus (Bath). b: reaction temperature 40°C

Reactivation temperature (°C)

◆ 48

● 45

○ 37

△ 30

observed. When acetylene inactivated cells were concerned, the pH did not decrease following the addition of methanol. One reason for this, is an accumulation of formic acid caused by the inactivation of formate dehydrogenase resulting from the production of PO (Tatsuka and Kamata, personal communication). By controlling the pH at 7.8, some experiments proved successful with regard to reactivating cells using methanol as a carbon source (Fig. 7.3.3). However, in these cases, a very long lag period and a slow reactivation rate was observed. The reasons for this phenomenon were not clear at the time. However, by changing the method of the "cultivation of the cells", in this case, the carbon source, methanol was used instead of methane, the reactivation process was facilitated. Cells were cultivated under methanol-limited conditions.

By using methanol as a carbon and energy source for the reactivation of cells, the lag period which was observed when methane was used for this purpose, had disappeared (Fig. 7.3.4) and the initial reactivation rate was increased (Fig. 7.3.5). What is certain is that the cells can metabolise carbon source more easily for reactivation when methanol is a carbon and energy source. The mechanism of decrease in pH and inhibition of reactivation when methanol was used as a carbon and energy source for the reactivation of methane-grown cells will be discussed later in Chapter 9.

7.3.4 The Effect of Copper on the Reactivation of Propylene-inactivated Cells

The evidence of reversible inhibition of particulate MMO by metal chelating agents such as thiourea, indicates that copper is involved in the activity of particulate MMO. If this system is inactivated following the production of PO, copper appeared to be required for reactivation.

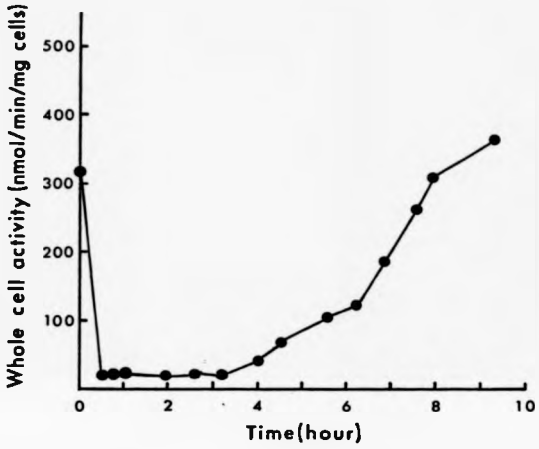


Fig. 7.3.3 The effect of methanol as the carbon source on the reactivation of propylene-inactivated cells of Methylococcus capsulatus (Bath)

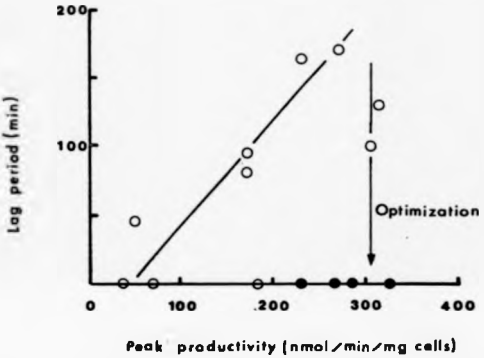


Fig. 7.3.4 The effect of peak productivity on the lag period observed during the initial period of reactivation in Methylococcus capsulatus (Bath)

○ Methane as the carbon source for the reactivation

● Methanol as the carbon source for the reactivation

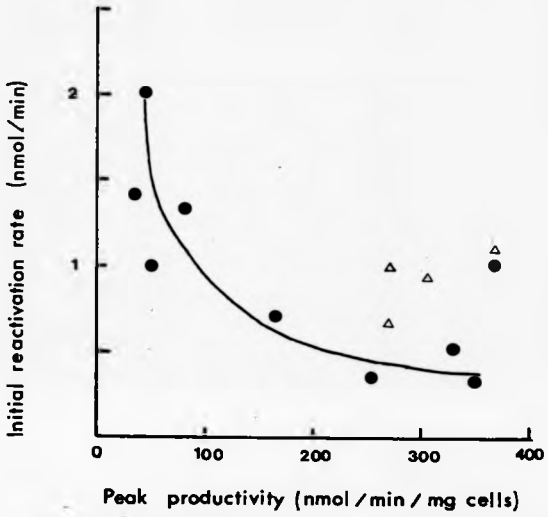


Fig. 7.3.5 The effect of carbon sources on the initial reactivation rate in Methylococcus capsulatus (Bath)

Carbon source for reactivation

● Methane

Methanol

7.3.4.1 Experimental

Cells which contained particulate MMO were collected from the chemostat culture grown on NMS medium containing 3 mg/l of $CuSO_4.5H_2O$. The cells were washed twice with copper-free NMS medium and then resuspended in the same medium. The cells were then inactivated in the presence of acetylene or propylene. After removing PO or acetylene, the cell suspensions were subdivided and 20ml of each sample was added to 250ml flasks. Different concentrations of copper sulphate were added to each flask and then closed with a Suba-Seal stopper. 50ml of methane was added to each flask and then incubated at a temperature of 45°C for Methylococcus capsulatus (Bath) and 30°C for Methylocystis parvus (OBBP).

7.3.4.2 Results and Discussion

The effect of copper ions on the reactivation of acetylene-inactivated cells of Methylococcus capsulatus (Bath) are shown in Fig. 7.3.6. Surprisingly, no difference was observed between 0 - 3 mg/l of $CuSO_4.5H_2O$. However, at 5 and 10 mg/l, the reactivation of the MMO was actually inhibited. Cells inactivated under conditions of PO production were inhibited much more, even at a low copper concentration (Fig. 7.3.7).

In Methylocystis parvus (OBBP), similar results were obtained (Fig. 7.3.8, 7.3.9). These results indicate that copper is not necessary for the reactivation of particulate MMO, indeed it contributes to the inactivation. The Wca of cells which were reactivated without copper, were still completely inhibited as a result of the addition of 1 mM thiourea. The non-requirement of copper for the reactivation of particulate MMO could be due to the following reasons:-

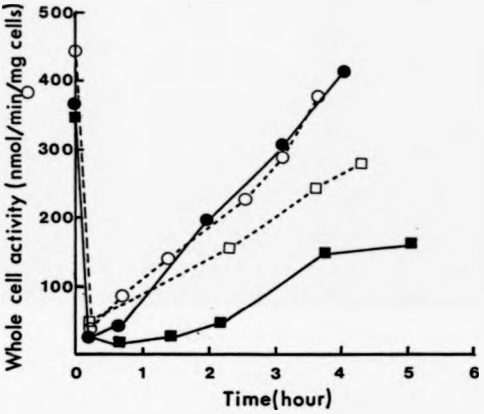


Fig. 7.3.6 The effect of copper ions on the reactivation of acetylene-inactivated cells of Methylococcus capsulatus (Bath)

Copper concentration (mg/l)

- ● 0
- ○ 1.0
- □ 5.0
- ■ 10.0

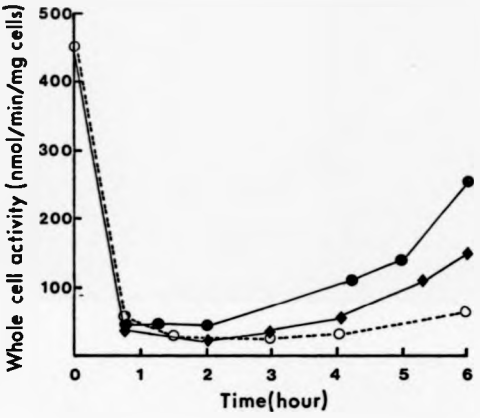


Fig. 7.3.7 The effect of copper ions on the reactivation of propylene-inactivated cells of Methylococcus capsulatus (Bath)

Copper concentration (mg/l)

● 0

◆ 1.5

○ 2.5

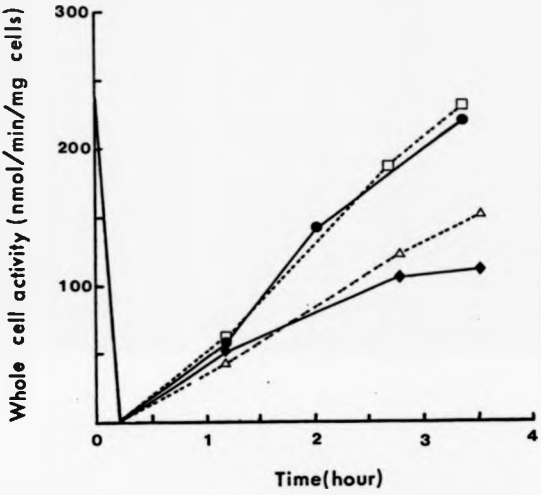


Fig. 7.3.8 The effect of copper ions on the reactivation of acetylene-inactivated cells of Methylocystis parvus (OBBP)

Copper concentration (mg/l)

- ● 0
- □ 1
- ▲ 2
- ◆ 8

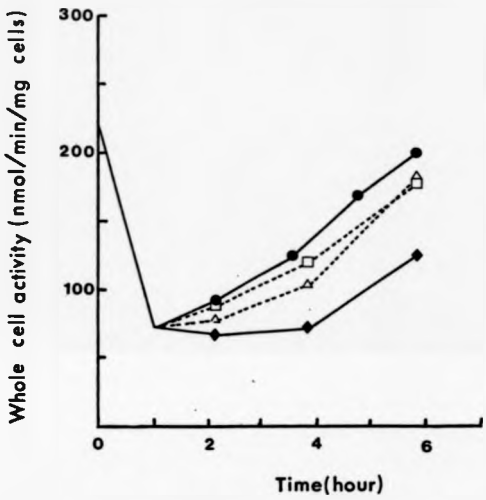


Fig. 7.3.9 The effect of copper ions on the reactivation of propylene-inactivated cells of Methylocystis parvus (OBBP)

Copper concentration (mg/l)

- ● 0
- △ 1
- □ 2
- ◆ 8

a) A part of the protein of MMO, which includes the active site is degraded and freshly synthesized during the reactivation process. Copper is not associated with this part of protein.

b) Particulate MMO is degraded completely. However, the copper associated with inactivated MMO is reabsorbed to form a freshly synthesized MMO.

c) The copper absorbed by the cells (but not by the MMO) is used to synthesize MMO.

Copper ions are necessary for the expression of particulate MMO, however its optimum concentration is related to the biomass concentration (Prior, 1985; see Chapter 3). The biomasses used in the experiments throughout this Section were relatively low. It was seen that at high copper concentration (above 3mg $CuSO_4.5H_2O$/l) inhibition of reactivation was seen. However, the reason for the non-requirement of copper for the reactivation cannot be well explained. When cells were growing during the reactivation process, it was recognized that the copper supply was necessary.

In the next Chapter, the mechanisms of inactivation and reactivation of the cells will be considered. These are important for the prevention of inactivation and acceleration of reactivation.

CHAPTER 8

Elucidation of Inactivation and Reactivation Mechanisms under the Conditions of PO Production

8.1 Introduction

The mechanisms of inactivation by external PO have been elucidated gradually, however the reason why cells are inactivated following the oxidation of propylene is still unsolved. It is considered that PO is related to the inactivation, although the inactivation mechanism is different to that of inactivation by external PO since the reactivation patterns does not resemble each other. In the production of PO, two inactivation mechanism are considered. These are inactivation of MMO and an unidentified inactivation process (concealed inactivation) following the production of PO, these are independent of external PO.

8.2 PO Concentration in the Cell under the Conditions of PO Production

8.2.1 Introduction

It appears that inactivation under conditions of PO production is occurring within the cells. There is a similar example which is the accumulation of ethanol within the cells has already been demonstrated. It is generally recognized that ethanol has three inhibitory (inactivating) effects. These are: the inhibition of cell multiplication; the inhibition of fermentation and a lethal effect on cells. PO demonstrates many similar effects on cells. It was reported by Nagodawithana and Steinkraus (1976) that when ethanol was added to a *Saccharomyces cerevisiae* culture, it was less toxic to the yeast cells than was ethanol produced by the yeast cells themselves. The reasons suggested for this phenomenon were: the depletion of nutrients (Casey *et al*., 1984; Dombek and Ingram, 1986); the formation of toxic by-product (Viegas *et al*., 1985) and the intracellular accumulation of ethanol during fermentation (Nagodawithana and Steinkraus, 1976; Beaven *et al*.,

1982; D'Amore et al., 1988). Applying these explanations to PO toxicity we can consider each in turn.

Firstly, the depletion of nutrients can be ruled out because the cells are capable of being reactivated after inactivation, in the same medium. Secondly, the possibility of there being a toxic by-product such as allylalcohol or formaldehyde will be discussed later in this Chapter. Thirdly, the writer of this thesis advances the theory that the PO which has accumulated in the cells inactivate them at the time of PO production. However to prove this hypothesis, it would be helpful to measure directly the precise level of PO in vivo. This is not a simple task, so by a modification of the IBF-reactor, a retention time of PO in the cell was measured and using this value, the intracellular PO concentration was estimated.

8.2.2 Experimental

To assume the retention time of PO in the cells is necessary for the calculation of the intracellular PO concentration. In order to estimate the mean retention time of PO, that is the period when PO is produced by MMO until PO is emitted to outside of the cell, the IBF-reactor was used. A preliminary experiment was set up. The effluent at the outlet of the IBF-reactor was collected every 5 seconds and samples were analyzed. It was found that the reaction was extremely rapid and PO was detected within 10 seconds after the propylene, methanol and oxygen were brought into contact with the cells. In order to measure the retention time of PO in the cell accurately, the method described below was used.

The IBF-reactor was set up as described before but was modified such that the effulument was connected to tygon tubing (1.5mm internal diameter) precalibrated for the liquid volume/tube length relationship.

The NMS medium which contained propylene and oxygen, but not methanol was supplied continuously to the IBF-reactor. This medium was replaced by the NMS medium which contained propylene, oxygen and methanol. The reaction mixture in the tube was carefully analysed by drawing samples directly with a syringe through the surface of the tube.

The lag time when the methanol was first detected until that time when PO concentration suddenly increased was calculated from the liquid volume and the pump flow rate. It was assumed that the PO produced in the MMO diffused evenly inside the cell. Moreover, the time required for the diffusion of the PO from MMO to the outside of the cell (PO retention time) was calculated as a half of lag time mentioned above (the diffusion speed of methanol from outside of the cell to MDH was assumed the same of that of PO from MMO to outside of cell).

8.2.3 Results and Discussion

The period when methanol was first detected until the time when PO concentration suddenly increased, was only 5 seconds, so that the retention time of PO within the cell was calculated as 2.5 seconds. The PO concentration inside each cell could be calculated as follows:- if productivity was 500 nmol PO produced/min/mg cells (mU/mg cells) then 21 nmol of PO was produced in 2.5 seconds. If 1 mg of cell has a capacity (volume) of 1 μl which could retain PO within the cell, the amount of 21 nmol per 1 microlitre means 21 mM. Actually each cell is much smaller than 1 microlitre, however the above concept can be applied for the small cells. Thus, the concentration of PO in the cell reaches 21 mM when peak productivity is 500 mU/mg cells after the reaction has commenced. The concentration is totally dependent on PO productivity. Fig. 8.2.1 shows the relationship between the PO productivity and the PO

concentration in the cell. Fig. 8.2.2 shows the PO production curve obtained using IBP-reactor and the assumed change in intracellular PO concentration. The intracellular PO concentration during the early stage of the reaction, especially within the initial 10 minutes, was high and then gradually decreased with the decreasing PO productivity. The inactivation curve demonstrated tailing, this is indicative of lowered lethal damage at low productivity.

Many factors could have an effect on the intracellular concentration of PO, i.e. the active transport system of PO and propylene if any, diffusion, membrane structure, location of MMO etc. The last of these factors (the location of MMO) could be particularly important in the accumulation of PO during production. Type I and Type II methanotrophs have a different internal membrane structure, furthermore, the location of particulate MMO and soluble MMO may be different. The discharge of PO produced by MMO which was located on the external membrane appeared to be much faster than that of PO produced by MMO which was located on the internal membrane. However, these results on the accumulation of PO leave no doubt that PO is accumulated intracellularly.

With regard to the intracellular ethanol accumulation the discrepancies in the results from the various studies are thought to be primarily due to the controversy surrounding the accuracy of techniques used to measure intracellular ethanol concentration (Dasari et al., 1984; Dombek and Ingram, 1986). In recent years D'Amore et al. (1988), demonstrated that the intracellular accumulation of ethanol occurs during the early stages of fermentation.

The ethanol productivities reported by Moulin et al. (1984) and Brown et al. (1984) ranged from 1 to 4g ethanol/hour/g cells in Saccharomyces cerevisiae. In this thesis the productivity of PO using Methylococcus capsulatus (Bath) varied between 1 - 2.6 g PO/hour/g cells

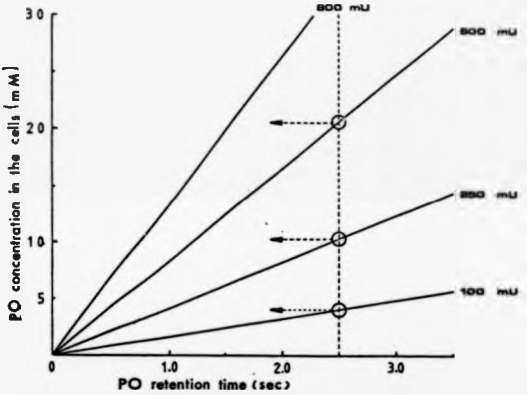


Fig. 8.2.1 Estimated intracellular PO concentration during the biotransformation of propylene

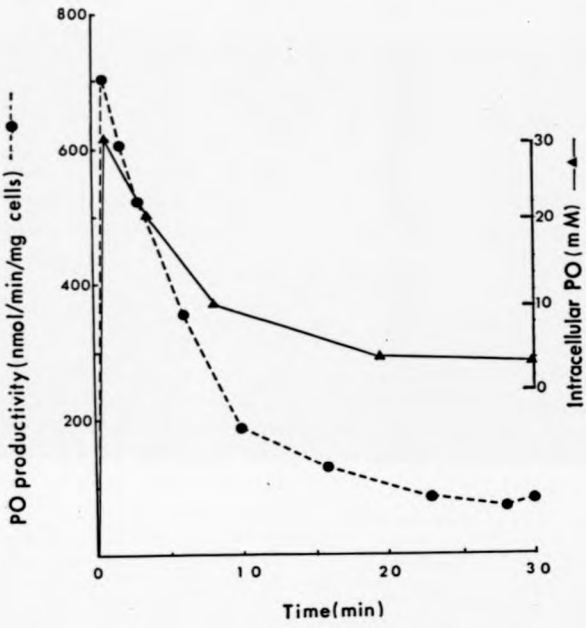


Fig. 8.2.2 Sequential PO production and the change of estimated intracellular PO concentration

(300 - 750 mU/mg cells). Ethanol may show the same intracellular mode of action in Saccharomyces cerevisiae as PO does in Methylococcus capsulatus (Bath).

If cells were inactivated by the intracellular PO, how does the PO act on the cells? Inactivation of MMO by PO can easily be understood, but the mechanism of inactivation by PO on the other systems such as formaldehyde dehydrogenase and methanol dehydrogenase has not been made clear. The possible inactivation mechanism by intracellular PO on the MMO and unidentified cause of inactivation will be discussed in the next Section.

8.3 Possible Inactivation Mechanisms of MMO by Intraceullar PO

In this Section, the similarity in the mechanisms of inactivation of MMO by external PO and PO formed at the MMO active site is discussed. Early experiment (see Chapter 6) showed that blocking the active site of MMO from externally added PO using methane or ammonia appeared to be effective in the prevention of inactivation. On the basis of above hypothesis, the effect of the addition of methane and ammonia on the PO productivity were studied.

8.3.1 Experimental

The effect of competitive substrates on inactivation of MMO was investigated using the immobilized cell bio-film reactor (IBF-reactor), as described in Chapter 5.

8.3.2 The Effect of Methane on Inactivation of Cells

Fig. 8.3.1 shows the effect of methane on the inactivation under

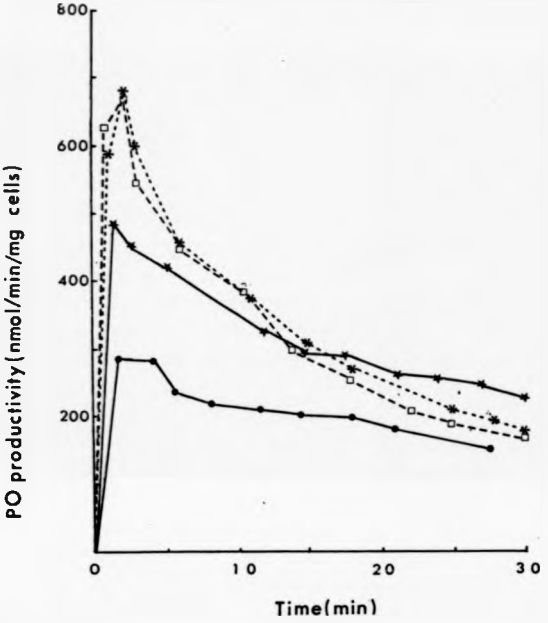


Fig. 8.3.1 The effect of methane on the inactivation of cells under conditions of PO production in the IBF-reactor

Gas composition			
	Propylene	Oxygen	Methane
□	50	50	0
✱	47.5	47.5	5
★	42.5	42.5	15
●	28.75	42.5	28.75

conditions of PO production. Increasing the methane concentration caused a depression in peak productivity and the inactivation rate was decreased, however no significant protection effect was observed.

8.3.3 The Effect of Ammonia on Inactivation

The first report of ammonia oxidation by a methanotroph was by Hutton and Zobell (1953), subsequently Dalton (1977) reported the oxidation of ammonia by the cell extracts of Methylococcus capsulatus (Bath). Ammonia oxidation was 4% of the methane oxidation rate as measured by oxygen uptake in Methylosinus trichosporium (OB3b) (O'Neill and Wilkinson, 1977).

When ammonium chloride was added to the reaction medium, peak productivity was reduced and the rate of inactivation was also decreased (Fig. 8.3.2). However, this prolongation of the half-life appeared to be related to the decreased peak productivity due to competition between propylene and ammonia rather than a protection effect of ammonia.

8.3.4 Discussion

When ammonia or methane was added to the reaction medium, the peak PO productivity was reduced. However, these inactivation patterns in the IRF-reactor were slightly different from those of oxygen or electron donor-limited conditions. In the latter cases, the peak productivity (not representing the potential cell activity) did not decrease until the limitation was relieved. By contrast, when methane or ammonia was added, productivity decreased immediately after the peak productivity. It was considered that peak productivity was reduced by the competition with propylene, however MMO was always working at its maximum capacity.

The ineffectiveness of ammonia or methane to prevent inactivation

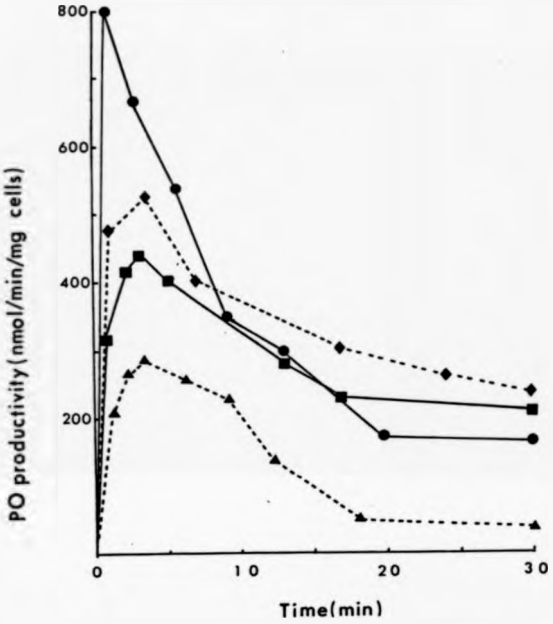


Fig. 8.3.2 The effect of ammonia on the inactivation of cells under conditions of PO production in the IBF-reactor

Ammonium chloride (mM)
- ● 0
- ◆ 5
- ■ 10
- ▲ 20

under the conditions of PO production suggests a mechanism for the mode of action of PO in the inactivation of MMO. PO added externally to the cell suspensions may inactivate the active site of MMO by a mechanism similar to that of acetylene. In this case PO enters into the active site from an external source. The mechanism of inactivation of MMO under the conditions of PO production was initially considered to be identical. That is, the PO produced in the active site of MMO is released, then it re-enters into the active site as a substrate. This hypothesis is incorrect because ammonia or methane are not able to protect against inactivation under the conditions of PO production. Whereas they are effective protectors when externally added PO is used to inactivate (Stanley and Richards, unpublished data). The affinity of methane and ammonia for MMO must be higher than PO nevertheless methane and ammonia cannot protect under the conditions of PO production indicating that the PO which inactivates the active site of MMO is not coming from the outside. The PO formed in the MMO active site remains there and is then further oxidised by MMO to cause inactivation. Fig. 8.3.3 shows possible routes of inactivation by PO.

The loss of activity is faster under the condition of high PO production, especially in those with more than 200 mU/mg cells productivity, and when productivity declines the rate of loss of activity also decreases (see Chapter 5, 7). It is suggested that at the active site, there is some microenvironment to prevent the release of PO from the site of its formation under the conditions of high PO production.

The prevention of inactivation of MMO using competitive substrates appeared to be less effective. The idea to release PO from the active site more quickly should be applied. This will be discussed in the General Conclusions (Chapter 11). In the next Section, the mechanism of concealed inactivation which was assumed from the results of the

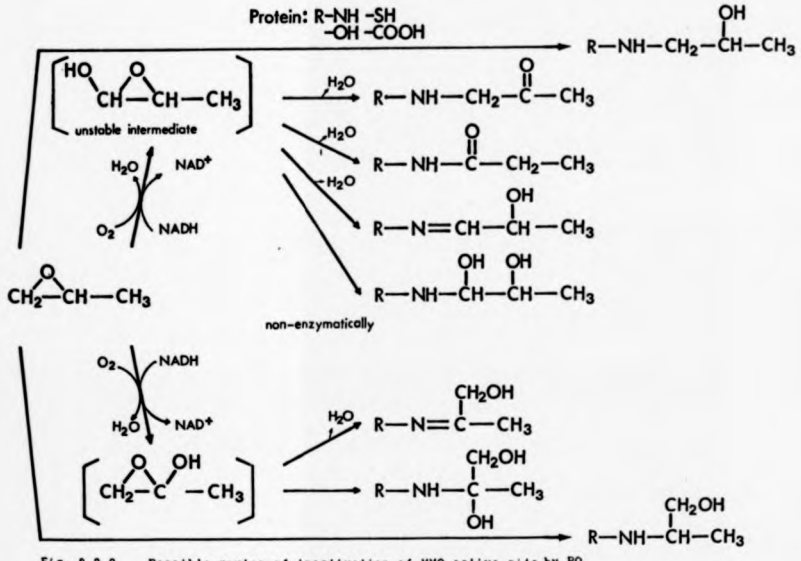


Fig. 8.3.3 Possible routes of inactivation of MMO active site by PO

reactivation of propylene-inactivated cells will be discussed.

8.4 Possible Inactivation Mechanisms of Concealed Inactivation

With regard to the mechanism of the concealed inactivation, there are several possibilities which have not been proposed in previous literature, these are:- a) inactivation by autolysis, b) inactivation by toxic by-product, c) inactivation by a solvent-like effect of PO, d) inactivation by formaldehyde which is an intermediate of methanol as an electron donor.

8.4.1 Inactivation of Cells by Autolysis

Organisms possess a variety of enzymes which are capable of degrading their own biological materials to maintain a constant pool of useable substrates. These include for example, proteases, in particular which are responsible for the turnover of proteins. If the inactivation of the biocatalyst was caused by some enzymes such as a protease or an esterase where activity is usually depressed but is activated by the oxidation of propylene. To confirm this possibility of autodegradation, a reaction was set up intermittantly using the IBP-reactor. The result is shown in Fig. 8.4.1. Methylococcus capsulatus (Bath) was used for this experiment.

When the reaction was commenced, by supplying the medium containing propylene, oxygen and methanol, the cells lost their activity. A few minutes later, when the reaction was ceased by changing to the medium without propylene, oxygen and methanol, the inactivation observed also ceased. This intermittant reaction was continued until the reaction period (total of the periods during which the reaction medium was supplied) reached 30 minutes. The inactivation patterns of the

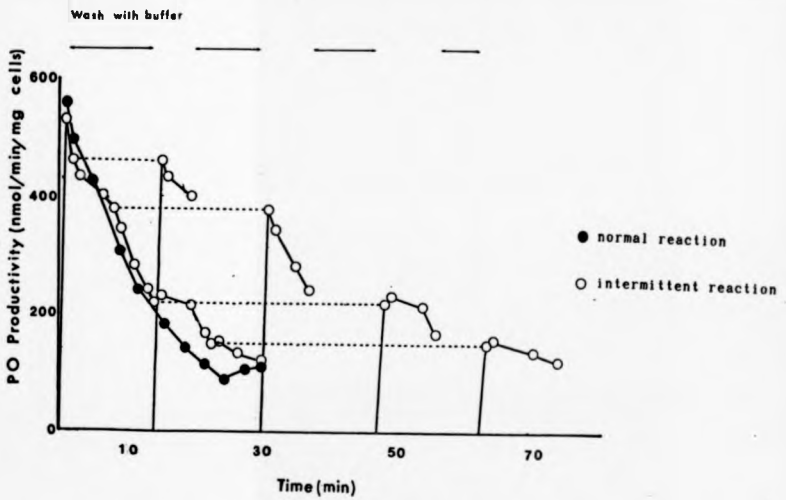


Fig. 8.4.1 Intermittent reaction and the inactivation of cells of Methylococcus capsulatus (Bath)

intermittent reactions in which the washing period (period when the medium without propylene and methanol was supplied) was eliminated and a continuous reaction was compared (Fig. 8.4.1).

The inactivation of cells was found to be limited to the reaction period only and no inactivation was observed during the washing period. This result indicates that there is no evidence on the inactivation by a protease-like degradation enzyme and that inactivation is attributed to the oxidation of propylene itself. In addition, if inactivation is dependent on a toxic compound which is accumulated during the reaction in the cell, this compounds must be washed out easily from the cells.

8.4.2 By-product Formation and Inactivation during the Oxidation of Propylene

If propylene is oxidised to a toxic compound other than PO, it may be this other product which inactivates the biocatalyst. Several compounds can be proposed as candidates these are allylalcohol and its derivatives (acrolein, acrylic acid), glycidol, 2-hydroxy propylene. MMO catalyses hydroxylation reactions (see Chapter 3) such that if the methyl-moiety of propylene was oxidised by MMO, allylalcohol would be produced. Allylalcohol is a known inactivator of whole cell MMO activity (Stanley and Richards, unpublished data). Allylalcohol was further oxidised at a rate of greater than 800 mU/mg cells by both Methylococcus capsulatus (Bath) and Methylocystis parvus (OBBP) to the corresponding aldehyde and acid (data are not shown). However, neither allylalcohol nor acrolein was detected following the oxidation of propylene by the above organisms.

Glycidol is another possible by-product of the propylene oxidation. There are two possible ways that glycidol is produced by MMO. One is from allylalcohol and the other is from PO. As mentioned in Chapter 3,

allylchloride was oxidised by MMO and epichlorohydrin was formed. However glycidol was not detected following the oxidation of propylene by Methylococcus capsulatus (Bath) and Methylocystis parvus (OBBP) both of which contained particulate MMO. To determine whether allylalcohol, or other toxic compounds could cause inactivation if produced from the oxidation of propylene, an indirect method was used.

Ethylene has no methyl substitute in its molecule, so ethylene oxide is the only product. Using the IBF-reactor, inactivation of cells following the oxidation of ethylene was tested with Methylococcus capsulatus (Bath). There was no significant difference in the inactivation of cells either oxidising propylene or ethylene (Fig. 8.4.2). This result supports the view that cells are inactivated following the epoxidation of a double bond.

8.4.3 How Does Propylene Oxide Act as an Inactivator within the Cells under Conditions of PO production?

The causes of concealed inactivation of cells under the conditions of PO production are not yet clear. One possibility is that intracellular PO may act as a solvent. For example, hydrocarbon solvents and their derivatives, such as benzene or short chain alcohols are known to denature both proteins and membranes. If the intracellular concentration of PO at the time of oxidation of propylene is high as predicted in the previous Section, then PO may act so as to have a solvent-like effect and denature protein or other cell components. Many solvents are oxidised by methanotrophs (see Chapter 3) and provide toxic products. In order to investigate its solvent-like effect on cells, benzene was selected, because particulate MMO cells cannot oxidise aromatic compounds (Prior and Dalton, 1985a). Hence it could be studied without any formation of a toxic product.

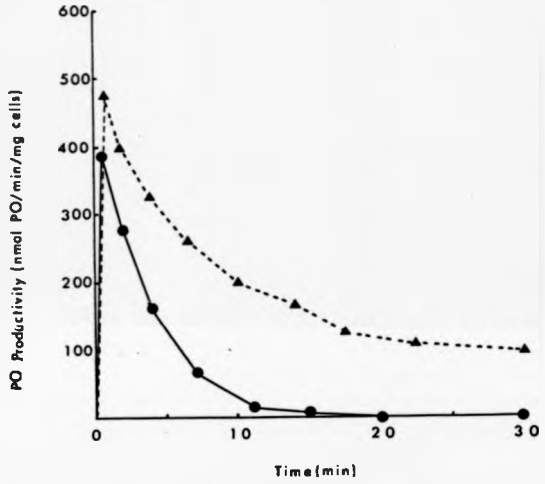


Fig. 8.4.2 The inactivation of cells following the oxidation of ethylene in Methylococcus capsulatus (Bath)

Inactivation of cells following the oxidation of propylene (▲), or ethylene(●).

8.4.3.1 Experimental

Benzene and acetylene were used as the cell inactivator. PO and propylene were also used to compare the inactivation- and reactivation-patterns. Cells containing particulate MMO were subjected to inactivation by benzene and acetylene which were then removed from the culture by evaporation when 20% methane was supplied at a rate of 1 VVM. PO was removed by scrubbing with air at a rate of about 30 VVM. After removing the inactivators, the cells were kept under conditions of reactivation with methane (20%) as the carbon source. Both the activity of MMO and methanol dehydrogenase (MDH) were monitored during the reactivation process. Methylocystis parvus (OBBP) was used throughout the experiments.

8.4.3.2 Results and Discussion

About 70% of whole cell MMO activity and 15% of MDH activity were lost following the production of PO in Methylocystis parvus (OBBP) (Fig. 8.4.3). The reactivation rate of MDH activity was greater than 2 times more rapid than that of the Wca. When, however, the cells were treated with the addition of acetylene or external PO (3mM), only the Wca was inactivated in Methylocystis parvus (OBBP) (Fig. 8.4.4). The reactivation patterns of both acetylene-inactivated cells and 3 mM PO-inactivated cells closely resembled each other. However, when the cells were treated with 30 mM PO, the inactivation and reactivation pattern changed. The pattern assumed a much closer similarity to that of PP-inactivated cells (Fig. 8.4.5).

Benzene was observed to have a distinct effect on both Wca and MDH activity. One pulse addition of 2.5 mmol (5.0mM) benzene fed to 500 ml cell suspension did not produce any effect on either MMO or MDH

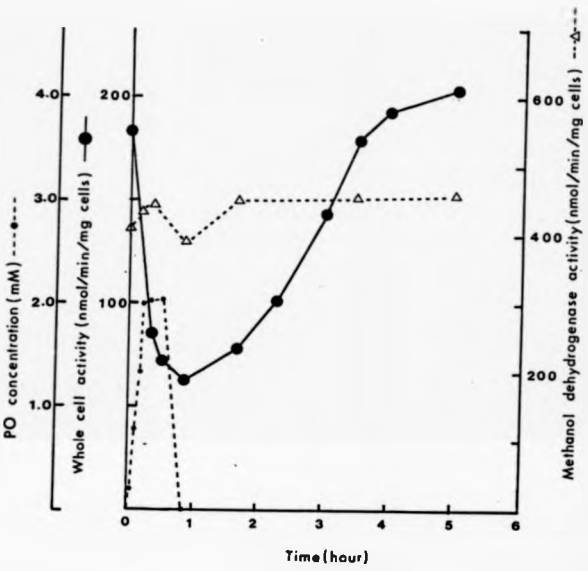


Fig. 8.4.3 The inactivation of enzymes under the condition of PO production in Methylocystis parvus (OBBP)

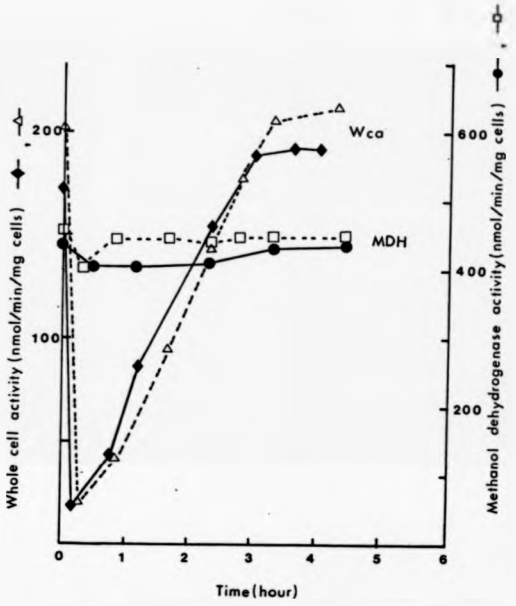


Fig. 8.4.4 Inactivation of enzymes by the addition of acetylene or propylene oxide and their reactivation in Methylocystis parvus (OBBP)

◆ ● Acetylene-inactivated cells

▲ □ PO-inactivated cells

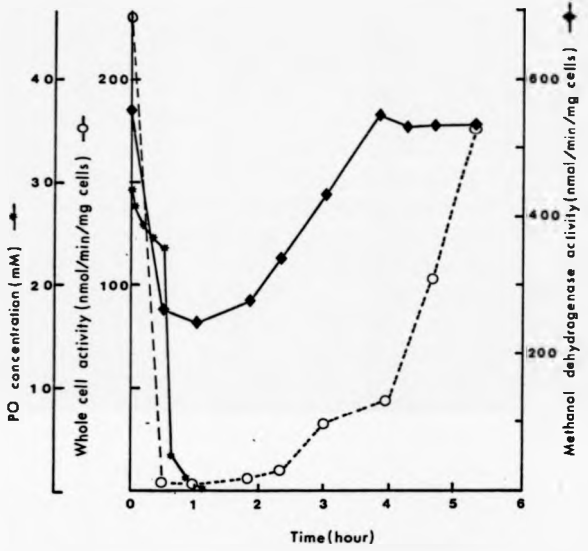


Fig. 8.4.5 The inactivation of enzymes following the addition of high PO concentrations and their reactivation in Methylocystis parvus (OBBP)

activity. However, when 3.75 mmol (7.5mM) of benzene was added, the whole cell MMO activity was decreased but the MDH was not (Fig. 8.4.6). The inactivation of whole cell MMO activity effected by benzene was different from that of inactivation brought about by extracellular PO. The cells lost their activity much more slowly than those of PO-treated cells and even when the benzene had disappeared, the cells continued to lose their activity for a short period of time. The reactivation rate of the Wca was similar to that of acetylene-inactivated cells.

However, when 5 mmol of benzene was added to the 500ml cell-suspensions (10mM) the inactivation and reactivation patterns were drastically changed (Fig. 8.4.7). The inactivation of Wca and MDH activity closely resembled that of cells which were inactivated under the conditions of PO production as shown in Fig. 8.4.3. Moreover, a lag period at the beginning of the reactivation process was also observed. These results support the view that intracellular PO accumulated under the conditions of high PO production, a condition which inactivated not only MMO but also other systems by their solvent-like effect.

8.4.4 The Effect of Electron Donors in Inactivation

So far, methanol was used as an electron donor throughout the inactivation and reactivation studies. Methanol is a substrate of MMO being converted to formaldehyde which may inactivate the biocatalyst. To investigate the effect of different electron donors on the inactivation of cells, formaldehyde and formate were supplied instead of methanol.

8.4.4.1 Formaldehyde

When 1.5 mM formaldehyde was supplied as an electron donor to the

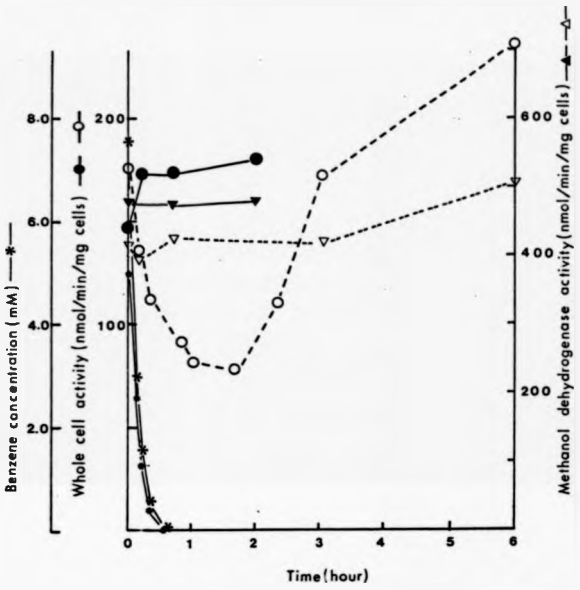


Fig. 8.4.6 The inactivation of enzymes following the addition of benzene and their reactivation in Methylocystis parvus (OBBP) (1)

Benzene concentration (mM)

5.0 Wca ●
MDH ▼

7.5 Wca ○
MDH ▽

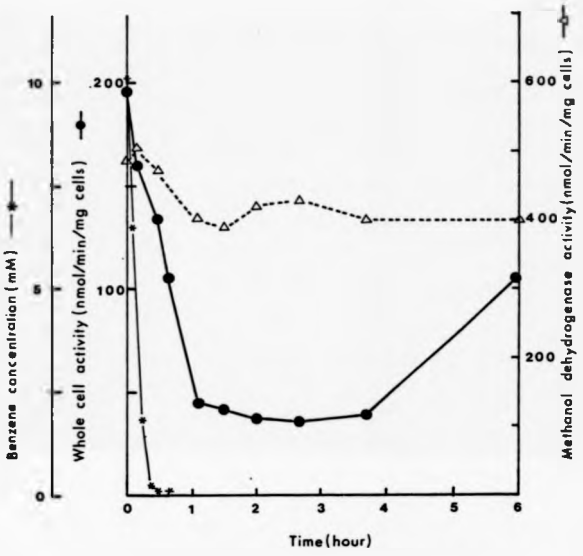


Fig. 8.4.7 The inactivation of enzymes following the addition of benzene and their reactivation in Methylocystis parvus (OBBP) (2)

IBF-reactor, the peak productivity was depressed compared to the peak productivity obtained using 0.3 mM methanol. To demonstrate the loss of potential activity during the production of PO, the following experiment was done. Methanol (0.05 mM) and formaldehyde (1.5 mM) were supplied as electron donors for 30 minutes (both electron donor limitation conditions), because these two concentrations were known to provide the similar productivity (about 100 mU/mg cells). After 30 minutes each medium was replaced with the medium containing 0.3 mM methanol to give a residual potential activity of cells (non-electron donor limited condition). The potential activity of cells which were supplied formaldehyde was obviously lower than that when 0.3 mM methanol was supplied (Fig. 8.4.8). Methanol was less toxic than formaldehyde as an electron donor.

The result demonstrated above provided limited information on the effect of formaldehyde on the catabolic pathway enzymes. In order to investigate the relationship between formaldehyde and the concealed inactivation, cells were inactivated using formaldehyde. The reactivation pattern was monitored using a 700ml fermenter. When formaldehyde was added to cell suspensions of Methylococcus capsulatus (Bath) giving a final concentration of 10 and 12.5 mM. Under these conditions the cells were inactivated and their reactivation was strongly depressed (Fig. 8.4.9). In Methylocystis parvus (OBBP), the addition of 10 mM of formaldehyde inactivated both MMO and methanol dehydrogenase activity (Fig. 8.4.10). However, MDH was reactivated two times faster than that of MMO which had been inactivated by acetylene. The reactivation rate of the Wca in which cells had been inactivated by the addition of formaldehyde was found to be low and the reactivation pattern closely resembled that of propylene inactivated cells.

However, under the conditions of PO production, Wca was inactivated but MDH was not so highly inactivated. This suggested that formaldehyde only played a minor role in the loss of biocatalytic activity during

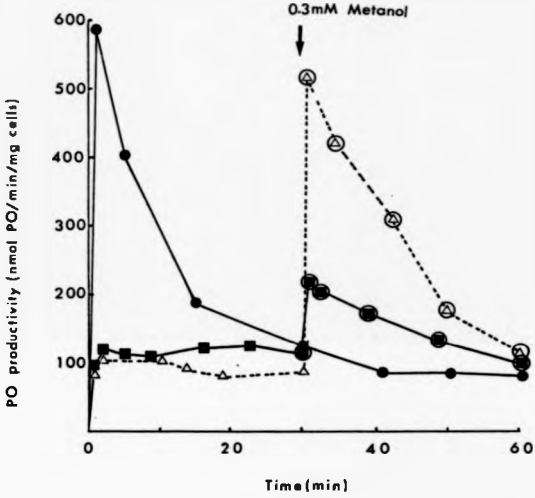


Fig. 8.4.8 The effect of formaldehyde as an electron donor on the inactivation of Methylococcus capsulatus (Bath) in IBF-reactor

Electron donor

- △ Methanol 0.05 mM
- ■ Formaldehyde 1.5 mM
- ● normal reaction

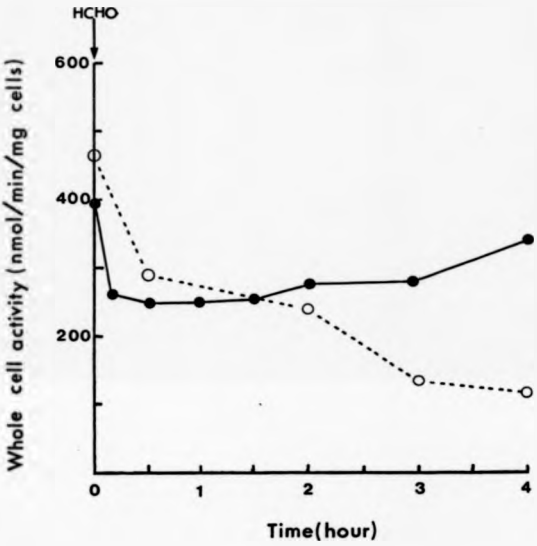


Fig. 8.4.9 The inactivation of cells following the addition of formaldehyde and their reactivation in Methylococcus capsulatus (Bath)

Formaldehyde was added at zero time at concentrations of 10 mM(●)or 12.5 mM (○).

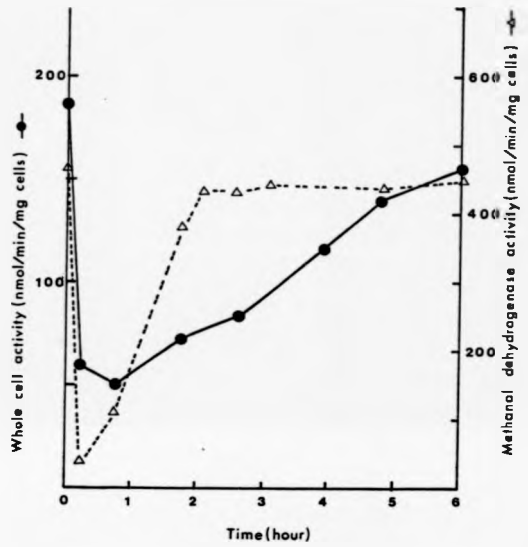


Fig. 8.4.10 The inactivation of enzymes following the addition of formaldehyde and their reactivation in Methylocystis parvus (OBBP)

propylene oxidation. The other results also support this hypothesis. When formate was used as an electron donor in the IBF-reactor, the cells were inactivated in a similar manner to that seen when formaldehyde was used as the electron donor (Fig. 8.4.11). From the above results, formaldehyde does not appear to be the major reason for the MMO and the concealed reactivation processes.

8.5 Comments

In this Chapter, it was proposed that the PO which was accumulated following the oxidation of propylene was the major cause of inactivation under the conditions of PO production. Furthermore, this inactivation effect of intracellular PO was thought to have two inactivation mechanisms, one was the inactivation of MMO and the other was the concealed inactivation which caused the delay of reactivation at the initial reactivation process as presented in Chapter 7. It was further proposed that the mechanism of the concealed inactivation by intracellular PO was a solvent-like effect of PO.

Many reports on cells or biological polymers have suggested that alkylation of DNA and RNA was the major cause of inactivation (Lawley and Jarman, 1972; Tamaratani et al., 1980). Tamaratani et al. (1980) demonstrated that breakdown products of DNA were lost during the reactivation of PO-treated spores. Salgveiro et al. (1988) also observed the leakage of 260-nm-light absorbing compound was dependent on the ethanol concentration in Saccharomyces cerevisiae. To determine whether a similar effect was observed in Methylocystis parvus (OBBP) the optical density (OD_{260}) of the supernatant of cell suspensions during the inactivation and reactivation was measured. The apparent increase in OD_{260} was observed in the PO-inactivated cell and propylene-inactivated cells (data not shown). However, the increase of OD_{260} was also observed in the acetylene-inactivated cells. This

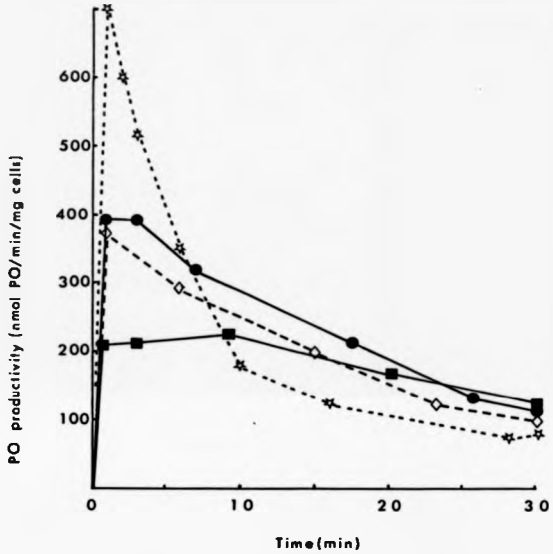


Fig. 8.4.11 The effect of formate as an electron donor on the inactivation of cells in the IBF-reactor

Formate concentration (mM)
☆ 0 (Control: 0.3 mM Methanol)
■ 25
● 50
◇ 75

suggests that the increase of OD_{260} may not be due to leakage of DNA fragment but due to the other compound such as cyclic-AMP in case of Methylocystis parvus (OBBP). Therefore DNA may not be alkylated in case of Methylocystis parvus (OBBP) at such a relative low PO concentration (up to 30 mM).

From the above results it is considered that the prevention of inactivation is very difficult, therefore the only way to develop the PO production process is thought to be the acceleration of reactivation of propylene-inactivated cells. In the next Chapter, in order to enhance the reactivation rate, factors of inhibition of reactivation will be considered.

CHAPTER 9

The Inhibition of Reactivation

9.1 Introduction

The prevention of inactivation and the acceleration of reactivation are both important for the development of the PO production process. Notably, the prevention of inactivation is more beneficial for the process because the low inactivation causes a rapid reactivation. However no effective prevention method has, as yet, been discovered. Before undertaking the study of the acceleration of reactivation, the inhibitors of reactivation were studied. Throughout the experiments on reactivation, a few factors have been thought to inhibit the reactivation. These were nitrite, methanol and formate but yet no reliable data had been obtained. In order to confirm the effects of above factors, both the IBF-reactor and fermenters were used.

9.2 The Effect of Nitrite on Inactivation and Reactivation

On several occasions, it had been observed that nitrite accumulated during the production of PO and also during the reactivation periods. In order to study the effects of nitrite on inactivation and reactivation, several experiments were undertaken.

9.2.1 The Effect of Nitrate on Reactivation

Firstly, the effect of nitrate on the reactivation of MMO was studied using Methylococcus capsulatus (Bath). Cells which had been inactivated by acetylene were washed twice with nitrate-free NMS medium using centrifugation. These cells were then resuspended in NMS medium and 20 ml of this suspension was put into several 250ml flasks. Various amounts of potassium nitrate were added. Then the flasks were sealed with a Suba-Seal, and 50 ml of methane was added by replacement of the

air. Flasks were incubated at 45°C.

The results are shown in Fig. 9.2.1. At the beginning of the reactivation process, rates of reactivation in the different concentrations of nitrate were similar but after the exponential stage, the reactivation rate was suppressed at concentrations of 2 g/l and 3 g/l even though cell growth remained constant. Following this result, the potassium nitrate concentration was kept to a level of 0.5 g/l in subsequent experiments.

9.2.2 The Inhibition of Reactivation by Nitrite

9.2.2.1 Experimental

The effect of nitrite on reactivation in Methylocystis parvus (OBBP) was investigated by adding potassium nitrite to the reactivation medium. The cells which had been inactivated by acetylene were washed twice with NMS medium (KNO_3 : 0.5 g/l) using centrifugation. They were then suspended in the same medium. These suspensions were then divided into 20 ml portions and put into 250 ml flasks each of which contained different amounts of potassium nitrite. To each flask 50 ml of methane was added, followed by incubation at 120 rpm and at a temperature of 30°C.

9.2.2.2 Results

The results of this experiment are shown in Table 9.2.1. By increasing the nitrite concentration, cell-growth was decreased but the reactivation rates were not inhibited even by the addition of nitrite up to a level of 1.5 mM, however, reactivation was inhibited at a nitrite concentration of 3.0 mM. It appears that a slight acceleration of the

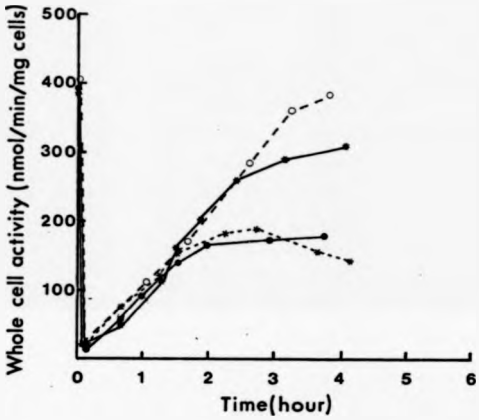


Fig. 9.2.1 The effect of nitrate concentration on reactivation in Methylococcus capsulatus (Bath)

Nitrate concentration (g/l)

- ○ 0.5
- ● 1.0
- ◉ 2.0
- ⊙ 3.0

Table 9.2.1

The inhibition of reactivation of acetylene-inactivated cells by the addition of nitrite

Nitrite concentration (mM)	Reactivation (3.5 hrs later) cell-growth [1] (OD_{540})	Wca [2] (nmol/min/mg cells)
0	2.40	168
0.5	2.34	179
1.0	2.12	204
1.5	1.92	202
3.0	1.88	132

1) Initial OD_{540} was 1.90

2) Initial Wca was zero

reactivation rate occurs with increasing nitrite concentration up to a level of 1.5 mM, which could be related to cell-growth and this will be discussed in Chapter 10.

The accumulation of nitrite during the inactivation and reactivation process appeared to be related to the inactivation or inhibition of nitrite reductase.

9.2.3 The Accumulation of Nitrite

In 1977, Stanley demonstrated that nitrite was accumulated when cells were inoculated into a medium which contained more than 0.8 g/l of potassium nitrate. This is the only known report relating to the accumulation of nitrite from nitrate in methanotrophs during the period of cell-cultivation. As previously described, a concentration of 3.0 mM of nitrite inhibited the reactivation of acetylene-inactivated cells and it also inhibited cell-growth. Furthermore, in the continuous PO production and reactivation process, it was sometimes observed that when nitrite was accumulated in the reactivator, reactivation of cells was inhibited. In order to avoid any accumulation of nitrite in the reactor and in the reactivator system, factors causing the accumulation of nitrite had to be investigated.

9.2.3.1 Experimental

Methylocystis parvus (OBBP) was cultivated continuously using methane as a carbon and energy source at 35°C. The culture was then diluted with NMS medium which contained 0.5 g/l potassium nitrate. 500 ml of this cell suspension was placed in the 700 ml volume fermenter. The cell suspension was kept at a pH of 7.0, temperature of 35°C and supplied with 20% methane in air at a rate of 1 VVM, and agitated at 500

r.p.m. The cells were then treated to stimuli such as acetylene, PO, propylene, or the process of varying the air supply after which the accumulation of nitrite was studied.

9.2.3.2 Nitrite Accumulation Following the Inactivation

When the cells were inactivated by acetylene, nitrite accumulated instantly, nitrate was present throughout the experiment (Fig. 9.2.2). By contrast, no nitrite accumulated under conditions where nitrate was absent in Methylocystis parvus (OBBP). Since acetylene is a suicide substrate of MMO, but not an inactivator or inhibitor of the nitrate reduction system, then this suggests that the accumulation of nitrite is not dependent on the inactivation of nitrate reduction enzymes. The nitrite accumulated in the cell suspensions was consumed gradually, following the reactivation of Wca.

Nitrite was also accumulated as the result of adding PO or propylene (Fig. 9.2.3.a, Fig. 9.2.3.b). In order to scrub the PO from the reactor, air was supplied at a rate of 20 VVM over a period of 20 minutes. As a result of this treatment, further accumulation of nitrite was observed. The absorption of nitrite and the reactivation of Wca were observed simultaneously.

9.2.3.3 The Effects of Oxygen and an Energy-supply on the Accumulation of Nitrite

It was assumed that the accumulation of nitrite was affected by dissolved oxygen (DO). The DO was controlled to a low level (below 5%) by using a high biomass and by controlling the agitation speed of fermenter. No nitrite accumulation was observed when the cells were inactivated by acetylene under conditions of oxygen-limitation (Fig.

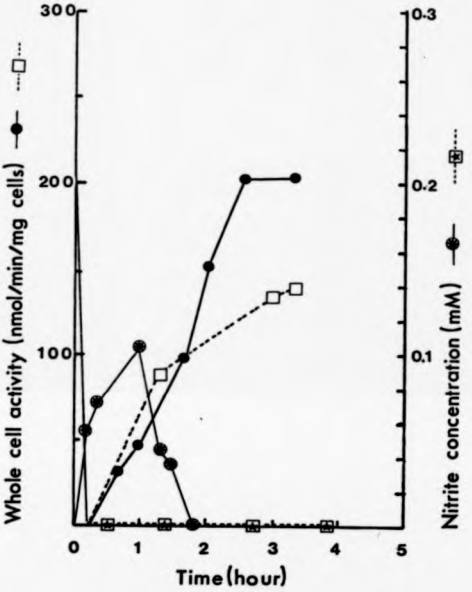


Fig. 9.2.2 The effect of nitrate on the accumulation of nitrite and reactivation in Methylocystis parvus (OBBP)

Nitrogen source

○ ● sodium nitrate

□ ⊞ dinitrogen

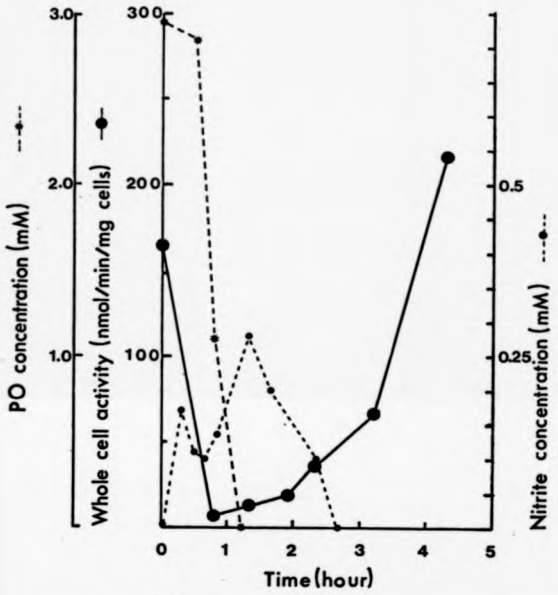


Fig. 9.2.3a The accumulation of nitrite following the addition of PO in Methylocystis parvus (OBBP)

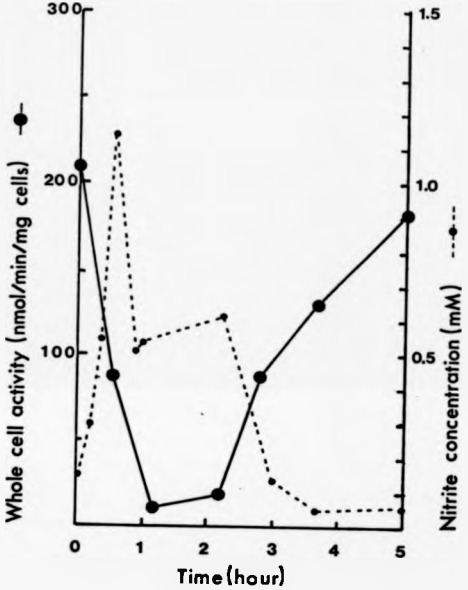


Fig. 9.2.3b The accumulation of nitrite following the production of PO in Methylocystis parvus (OBBP)

9.2.4). Nitrite accumulation was observed, however at higher DO value (greater than 90% by supplying air at 10 VVM with no carbon source) but this did not cause inactivation of MMO (Fig. 9.2.5). By substituting 20% methane in air for air alone (0.5 VVM), the nitrite concentration increased on one occasion, then decreased. Oxygen therefore appeared to act as a stimulus in nitrite accumulation. However, the time-lag observed after the increase in air supply (Fig. 9.2.5) suggested that oxygen (DO) may not be a direct cause of the accumulation of nitrite. Energy-starvation was considered as another possible cause of the accumulation of nitrite during the production of PO and during the reactivation process.

The addition of acetylene, propylene or PO to the cell suspension accelerated the consumption of energy. The reduction of nitrate and nitrite by their respective reductases also required energy as NAD(P)H. If the cell is deficient in energy this could lead to an accumulation of the intermediate, nitrite. Several reports on the relationship between energy and stability of the nitrate reduction system have been demonstrated. Kadam *et al*. (1974) found that when nitrate reductase was treated before hand with NADH, it was protected from inactivation caused by horseradish peroxidase. Dunn-Coleman and Pateman (1977) indicated that a decreased level of NAD(P)H resulted in a rapid loss of nitrate reductase activity but that this loss could be reversed by the addition of NAD(P)H. It was noted that when the NAD(P)H level is low, then the nitrate reductase was inactivated by oxidation. Dunn-Coleman and Pateman (1978), further suggested that nitrate reductase activity was subjected to redox control, the enzyme being inactivated when there was insufficient NAD(P)H, and reversibly activated when increased amounts of NAD(P)H are generated. The results cited relate to nitrate reductase and not to nitrite reductase, however NAD(P)H may also stabilise the nitrite reductase. A balance of energy on stability of nitrate

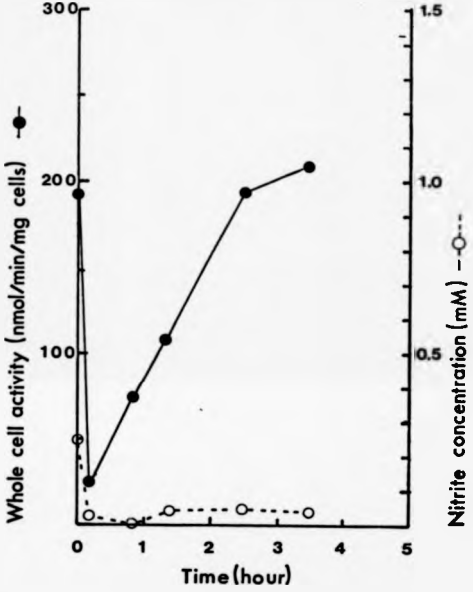


Fig. 9.2.4 The accumulation of nitrite following the addition of acetylene under the condition of low dissolved oxygen concentrations in Methylocystis parvus (OBBP)

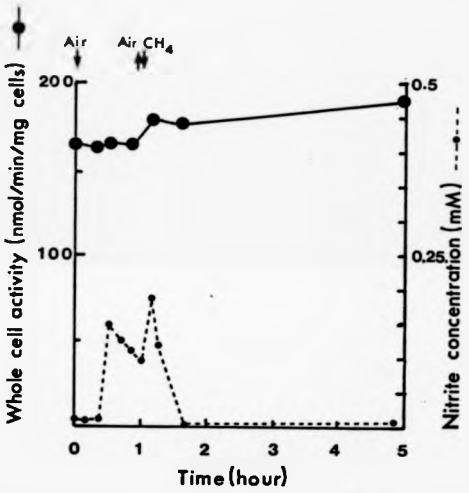


Fig. 9.2.5 The accumulation of nitrite following the treatment of high dissolved oxygen concentration condition in Methylocystis parvus (OBBP)

reduction enzymes may cause the accumulation of nitrite in the supernatant.

The consumption of nitrite following the reactivation of the cells can be interpretated as a requirement for a source of nitrogen for the process of reactivation and the supply of sufficient energy by the oxidation of the energy source.

The accumulation mechanism of nitrite is yet unsolved. However, throughout the studies on the accumulation of nitrite, the methods to avoid the accumulation of nitrite were found. To keep the dissolved oxygen at a low level and limiting the nitrate concentration are practices to be recommended.

9.2.4 Effect of Nitrite on the Production of PO

As mentioned above, nitrite acts both as a inhibitor of cell growth and for the reactivation of inactivated cells. Nitrite also inhibited MMO activity which may have an effect on the reactivation. The IBF-reactor was used to investigate the effect of nitrite on the MMO activity (production of PO) and on the process of inactivation. Sodium nitrite was added to the medium and fed to the IBF-reactor under non-limiting conditions.

As shown in Fig. 9.2.6, the peak productvity was reduced by the addition of nitrite at a level of 1mM. The effect of nitrite on PO production therefore appears to be similar to that observed with methane or ammonia. Methane and ammonia are substrates of MMO and competitors of propylene oxidation. However, there are no previous reports to show that nitrite is a substrate of MMO. If nitrite is not a substrate of MMO, in what other way is nitrite capable of reducing the peak productivity?

Recently, Carpenter *et al.* (1987) reported that clostridial ferredoxin and pyruvate-ferredoxin oxidoreductase were inhibited caused

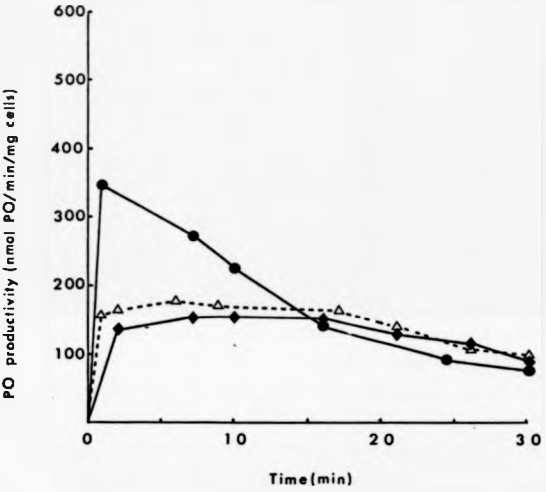


Fig. 9.2.6 The effect of nitrite on the production of PO in Methylococcus capsulatus (Bath)

Concentration of sodium nitrite (mM)

● 0

△ 1

◆ 3

by the binding of nitrite. Reddy et al. (1983) reported that the addition of sodium nitrite to suspensions of vegetative Clostridium botulinum resulted in the formation of iron-nitric oxide-complexes from the iron-sulphur centres of some or all of the iron-sulphur proteins present. It was concluded that since the iron-sulphur proteins are necessary for cellular metabolism then inactivation caused by the binding of nitric oxide would inhibit cell growth.

One possible explanation for the effect of nitrite on the depression of peak productivity is that the binding of nitrite to components of the electron transport system (such as protein C of soluble MMO) would cause an inhibition of the electron transport to MMO (protein A). The depression of peak productivity and the prolonging of the half life by adding nitrite suggest that the effect of nitrite is not that of inactivation, but of inhibition. These nitrite-induced changes appear to be similar to those conditions observed under electron donor- or oxygen-limited reactions (see Chapter 5).

As a result of the accumulation of nitrite, PO production in the reactor must be decreased. Moreover, reactivation rate may also be decreased because of the lowered oxidation rate of methane by the inhibition of MMO. Consequently this leads to a carbon and energy limitation for reactivation. Accordingly, the nitrite concentration both in the reactor and reactivator must be kept low.

9.3 Inhibition of Reactivation by Histidine

Histidine was also found as an inhibitor of reactivation. It has been known that some amino acids inhibit the cell growth of methanotrophs. Warner et al. (1983) reported that proline, threonine, methionine and lysine inhibited the cell growth of Methylosinus trichosporium (OB3b). The concentrations of amino acids which they used

were 2-4 mg/ml. As presented in Chapter 6, methionine (0.5 mg/ml) was effective as a sulphur source for the reactivation of MMO. Other amino acids such as glutamine, asparagine, casamino acids or peptone (0.5 mg/ml respectively) did not accelerate the reactivation rate but accelerated the cell growth (data not shown). Eccleston and Kelly (1972) reported that L-threonine, L-phenylalanine, L-histidine, L-tyrosine and L-homoserine inhibited the exponential growth of Methylococcus capsulatus (Foster and Davis) at 1.0 mM. Phenylalanine and tyrosine did not inhibit the reactivation and slightly accelerated in this thesis (see Chapter 11). However, histidine completely inhibited the reactivation of actylene-inactivated cells when 1 - 5 mM (0.15 - 0.75 mg/ml) was added to the cell suspensions of Methylocystis parvus (OBBP) which contained particulate MMO. It was also found that 5 mM histidine inhibited the Wca completely and this inhibition was reversible because when histidine-treated cells (5 mM) were washed three times with 4 mM phosphate buffer, 58% of activity was restored. Furthermore, by the addition of 40 μM of copper sulphate to the washed cells, 83% of the original Wca was restored.

Firstly, the inhibition effect of histidine was considered to be a competitor of propylene for MMO or inhibitor of methanol dehydrogenase. From its molecular structure, it was thought to be difficult that histidine could enter into the active site of MMO or methanol dehydrogenase. Now it can be understood that histidine chelates copper ions (II) and inhibits the electron transport of particulate MMO. Lau and Sarkar (1971) reported that there was an interaction of human serum albumin, copper (II) and L-histidine. They proposed that copper (II) bound to a dipeptide or tripeptide which included histidine in their molecules and regulated the transportation of copper (II) from blood to tissues (Lau and Sarkar, 1980, 1981; Lau et al., 1974). Brigarius et al. (1974) reported that low molecular weight

peptides, which include histidine in their molecules, chelated the copper ion and increased the superoxide dismutase activity. Furthermore, Weinstein and Bielski (1980) proposed the octahedral structure for the active complex $(Cu\ Hist_2\ H)^{3+}$ and showed that the copper-histidine complex was essential for the dismutation of the superoxide.

From the above information, it was considered reasonable that histidine chelated the copper which was associated with the particulate MMO and caused inhibition of its activity. Usually the concentration of histidine in the reactivator is low, so that reactivation may not be affected by histidine during the production of PO and/or reactivation of inactivated cells. However when the biomass concentration is high, the histidine concentration as well as other amino acids which may be released from cells should be monitored to ensure they do not accumulate and lead to inhibition of PO formation.

Throughout the study on the inhibition of reactivation, the only possible inhibitor which accumulated in the reactivator was thought to be nitrite. However, as already mentioned in this thesis, when methanol was used as a carbon source for the reactivation of propylene-inactivated cells, often no reactivation was observed. It was postulated that methanol or its derivatives might inhibit the reactivation of cells. In the next Section, the effect of methanol and formate on the reactivation of MMO will be discussed.

9.4 Is Reactivation Controlled?

Particulate MMO which has been inactivated by acetylene is reactivated *in vivo* by the addition of methane, methanol or formaldehyde. Hou *et al.* (1979d) proposed that methane was an inducer of MMO but it has been demonstrated in this thesis that methane was not an inducer of MMO synthesis. However, it has not yet been demonstrated

whether MMO synthesis is or is not controlled in cells. Dalton et al. (1984) have suggested that synthesis of the soluble MMO is probably induced by methane or methanol in Methylococcus capsulatus (Bath). They also noted that the regulation of particulate MMO might be different. Stanley et al. (1983) and Prior and Dalton (1985a) have demonstrated that the copper concentration is important for the expression of MMO activity in methanol-grown cells and that copper controlled the expression of particulate MMO. It appears possible by using the reactivation mechanism to determine whether particulate MMO synthesis is or is not controlled.

9.4.1 Experimental

Cells of Methylocystis parvus (OBBP) were cultivated using methane as a carbon and energy source, as described in Materials and Methods. The cells were inactivated by acetylene and then methanol or formate were added to the cell suspensions in order to investigate their effects on reactivation. During the period of reactivation treatment, methane was supplied continuously as a basal carbon and energy source for reactivation.

9.4.2 The Effect of Methanol on the Reactivation of MMO

To determine the effect of methanol on inactivation it was applied to cell suspensions over a 40 minute period. No inactivation was observed even when methanol was obviously detectable in the culture (Fig. 9.4.1). One pulse of acetylene (1 ml in 500 ml culture) was then added to the culture whilst the methanol concentration was maintained at a level of above 0.25 mM for a period of 2 hours. No reactivation was observed during the time that methanol was detectable, however methanol was

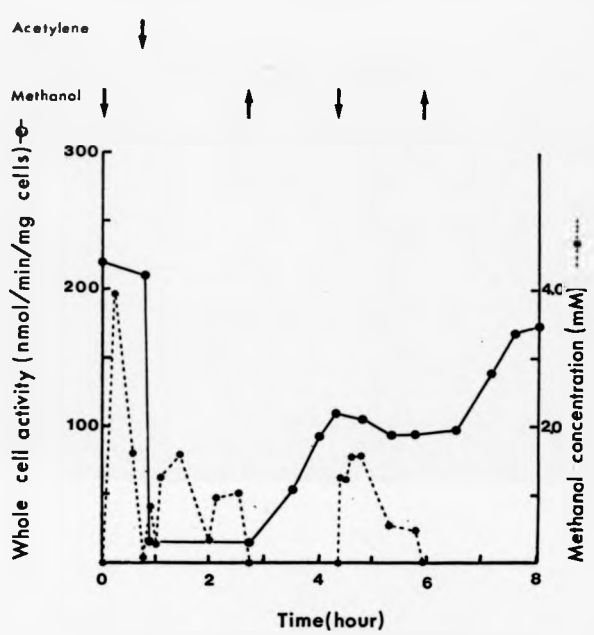


Fig. 9.4.1 The repression of reactivation of MMO by methanol in Methylocystis parvus (OBBP)

consumed by acetyene-inactivated cells throughout this period. When the methanol supply was cut off, it disappeared instantly from the culture supernatant and at the same time, MMO was reactivated. When Wca was partly reactivated it was observed that the inhibition of reactivation of Wca occurred immediately after methanol was supplied again. This result provides the first account concerning the repression of synthesis of particulate MMO by detectable amounts of methanol. Methanol did not inactivate MMO nor did it inhibit cell growth. However, it is yet not clear whether copper is an inducer of particulate MMO or not, because the cells inactivated by acetylene or PO do not require copper for their reactivation (see Chapter 7).

9.4.3 The Effect of Formate on the Reactivation of MMO

The repression of MMO synthesis may be due to the effect of secondary metabolites produced from methanol. One of these metabolites is formate. The effect of formate on the repression of MMO synthesis was therefore studied. To use the similar method as presented before in the effect of methanol on the reactivation, is difficult because the instant detection of formate (formic acid) and its control are not successful. Accordingly an another method was used. Cell suspensions which had been inactivated by acetylene were kept in a fermenter to which various amounts of potassium formate were added. Then 20% methane in air (0.5 VVM) was supplied continuously whilst pH was controlled at a level of 7.2 using 0.4M formic acid solution during the experiment. It was noted that formate depressed the reactivation rate of MMO, but did not repress it completely (Fig. 9.4.2). It was sometimes observed that formate accumulated during the production of PO or even after the reactivation treatment had started. Hence, the accumulation of formate in the reactivator is not favourable.

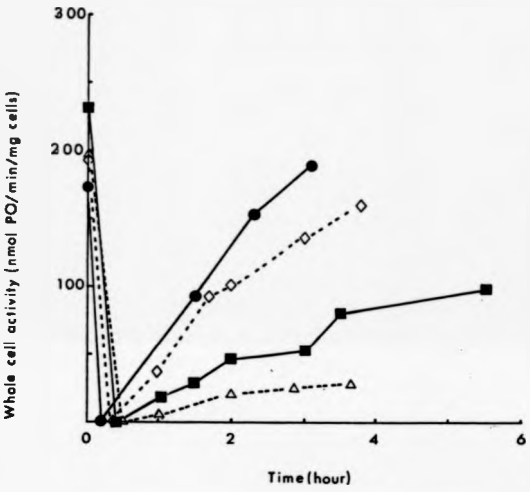


Fig. 9.4.2 The effect of formate on the reactivation of MMO in Methylocystis parvus (OBBP)

Amounts of formate added (mM)

● 0 (pH was controlled with HNO_3)
◇ 0 (pH was controlled with HCOOH)
■ 10 ( )
△ 30 ( )

9.4.4 Discussion

The major possible inhibitor of the reactivation was thought to be nitrite. The writer of this thesis recommended to keep the dissolved oxygen at a low level and limiting the nitrate concentration for avoiding the accumulation of nitrite. Another method to avoid the accumulation of nitrite is the usage of ammonia as a nitrogen source. Most methane-oxidising bacteria can use ammonia as a nitrogen source (Murrell, 1981). Organisms require less energy when ammonia is a nitrogen source compared to nitrate as a nitrogen source. Practically, ammonia was effective as a nitrogen source for the reactivation of acetylene- and propylene-inactivated cells of Methylococcus capsulatus (Bath) and Methylocystis parvus (OBBP) (data not shown). However, excess of ammonia in the culture also caused the accumulation of nitrite (O'Neill and Wilkinson, 1977; Dalton, 1977; Yoshinari, 1985; Pilkington, 1986). Accordingly, when ammonia is used as a nitrogen source, it must be carefully supplied.

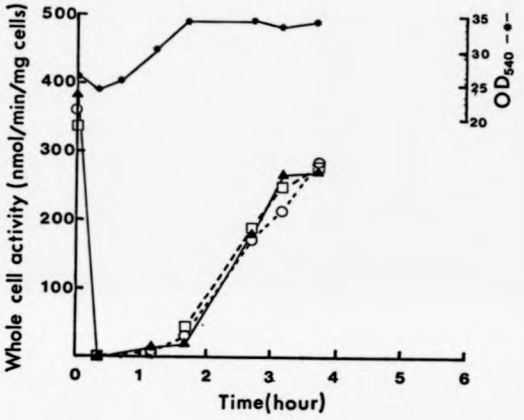
It was found that the MMO synthesis was repressed by a certain amount of methanol. Yet, as shown in Fig. 9.4.1, after the initial period of two hours repression, Wca was reactivated instantly when methanol was cut off. It seems quite certain that methanol per se represses the synthesis of MMO. However, there is a possibility that formaldehyde, the metabolite of methanol rather than methanol per se might repress MMO synthesis. Formaldehyde is a strong inactivator of both MMO and methanol dehydrogenase as shown in Chapter 8. However formaldehyde is also a carbon and energy source for the reactivation of cells if reasonable amounts (50 - 100 nmol/min/mg cells) are supplied continuously. From above observations, it is thought that methanol is the major repressor of the MMO synthesis.

The effect of methanol on the expression of MMO activity with

regard to methanol grown methanotrophs has for a long time been the subject of much controversy. However, a measure of agreement on this subject now appears imminent because of recent studies on the effect of copper on MMO activity by Stanley et al. (1983) and Prior and Dalton (1985a). The observation that the cells of Methylosinus trichosporium (OB3b) had no MMO activity when they are grown on methanol (Hou et al., 1979d) was probably due to the fact that cells were grown under conditions in which methanol was always present in excess in the culture. With regard to Methylocystis parvus (OBBP), when excess methanol was supplied, the cells were incapable of synthesizing MMO and finally the cells lost their MMO activity. This repression effect of methanol on MMO synthesis was also observed in Methylococcus capsulatus (Bath).

Methanol is more effective as a carbon source for reactivation than methane (see Chapter 6, 7), however, it also has a risk of a direct effect of inhibition of MMO synthesis. Moreover, excess methanol causes the accumulation of formaldehyde and/or formate and these compounds also inhibit the reactivation. Accordingly, when methanol is used as a carbon and energy source, it must be carefully supplied.

Throughout the studies on inactivation of cells by acetylene and its reactivation, one other phenomenon was observed. That is the instant ability of cells to grow on methanol (Fig. 9.4.3). Usually it is very difficult to cultivate methanotrophs on methanol as a carbon source without a long adaptation period. However, when the cells of Methylococcus capsulatus (Bath) and Methylocystis parvus (OBBP) in which MMO was inactivated by acetylene, cells grew very well on methanol. It was not necessary at all to adapt the cells to methanol. Acetylene-inactivated cells of Methylococcus capsulatus (Bath) and Methylocystis parvus (OBBP) could grow on NMS medium which contained 4% (v/v) methanol without any adaptation. This phenomenon could be



9.4.3 Adaptation of methane-grown cells to the methanol culture by using acetylene-inactivation technique

Electron donor
○ 1mM Methanol
▲ 2mM Formaldehyde
□ 100mM Formate

explained as follows. Methanol is a substrate of MMO being readily oxidised to formaldehyde (Colby et al., 1977). When normal cells are inoculated into fresh medium, containing methanol, these cells start to oxidise the methanol via MMO and methanol dehydrogenase to produce an excess of formaldehyde. The cell is incapable of oxidising all the formaldehyde produced by the assimilation and dissimilation pathways. Consequently formaldehyde begins to accumulate. This accumulation can lead to a rapid inactivation of many proteins within the cell by alkylation.

MMO-inactivated cells would have impaired methanol oxidising activity such that formaldehyde would be produced only by methanol dehydrogenase at a level that can be readily utilised by the formaldehyde-metabolising enzymes. Consequently this method for obtaining methanol grown methanotrophs is much simpler and less time-consuming than the long training method (Hou et al., 1979d) or even short adaptation method (4 days) obtained recently by Prior (1985) which use the gradual exchange of carbon sources from methane to methanol.

CHAPTER 10

Process Evaluation and the Economics of PO Production

10.1 Introduction

Nowadays, propylene oxide is produced chemically by the oxirane and the chlorohydrin processes. Before the introduction of the oxirane process in 1972, the chlorohydrin process was the only commercial process for the production of propylene oxide. The chlorohydrin process is still used and is responsible for approximately half of the world's total production of propylene oxide. Since these two processes are estimated to remain economically feasible in future, an evaluation of the bioprocess discussed in this thesis is made, by comparing it with the above mentioned processes.

Since the early 1980s, many studies on the economic viability of biological PO production processes have been undertaken. Hou, (1984a) suggested that the only way in which a process using methane-oxidising bacteria could be economical was if the surplus cells were to be used to fetch significant revenues as an animal feed. Drozd (1986) also suggested that in order to attain economic viability, the production rate of PO would have to be further increased and the problems of product-toxicity diminished. Moreover, it was realised that there would have to be a ready market for any biomass by-product. Such pessimism seems to derive from the apparent low productivity of PO and the instability of methanotrophs as a biocatalyst.

However, the conditions of the biological process using methanotrophs have been changed, by the development of cell cultivation-, reaction- and reactivation- technology as described in this thesis. The specific PO productivity (cell activity) of methanotrophs is not low when compared with the ethanol productivity by yeast, which is a typical commodity chemical produced by micro-organisms. The stability of methanotrophs as a biocatalyst for production of PO has not improved significantly, but by using the

reactivation mechanism, cells can be used repeatedly.

In this project, the PO productivity in the reactor has been achieved at a volumetric productivity of more than 100g PO produced/l/day at 15g biomass/l by developing the continuous two stage reactivation process at a scale of 0.35 l reactor - 8 l reactivator (Stanley and Suzuki, unpublished data). This productivity is 40 times higher than enzyme-based Catus-chlorohydrin bioprocess (see later in this Chapter) and at least 8 times higher than the direct oxidation bioprocess demonstrated by Hou et al. (1984b), Habets-Crützen et al. (1984), Furuhashi et al. (1981) and Miyawaki et al. (1986). Furthermore, a two stage reactivation process has been demonstrated to work for longer than 9 months continuously during this project.

In order to apply the reactivation mechanism to the continuous PO production process, the selection of a suitable strain is described first in this Chapter. Then in order to compare the process economics of biological- and chemical processes, the basic parameters were investigated. These are the stoichiometry of the methanol consumption and PO production and substrate consumption rate for reactivation. This will make the problems of the reactivation process clear showing which part of the process should be improved to compete with existing processes.

10.2 Selection of Suitable Organisms

10.2.1 Introduction

Most of the studies in this thesis have been conducted using Methylococcus capsulatus (Bath) because, as described briefly, a lot of information previously compiled on this organism is applicable to the development of PO production technology. In particular, Methylococcus

capsulatus (Bath) is able to grow at 45°C which provides a significant advantage for the recovery of PO from the reaction mixture. Since its boiling point is 34°C and at 45°C recovery can be made from the gas phase which has avoidable advantages over aqueous phase recovery systems. However, the stability and tolerance of above organism for PO is not as high as we expected. Dalton (1980) reported on the production rate of PO using different types of methanotrophs. From his results Methylomonas methanica seemed to be a more stable and tolerant strain for PO production. However, these were done in simple shake flasks where reaction conditions were not fully optimized. If the reactivation mechanism is common among methanotrophs, it may be possible to obtain a more suitable strain than Methylococcus capsulatus (Bath) for PO production.

Methanotrophs can be classified into three groups by their growth temperatures: these are called mesophilic methanotrophs, thermotolerant methanotrophs and thermophilic methanotrophs in this thesis. The mesophilic methanotrophs such as Methylosinus trichosporium (OB3b) or Methylocystis parvus (OBBP) can grow at 30°C but not at 40°C. The thermotolerant methanotrophs can grow at 45°C but not 50°C. Methylococcus capsulatus (Bath) and Methylococcus capsulatus (Texas) are included in this group. The thermophilic methanotrophs which can grow at temperatures greater than 50°C, these include Methylococcus thermophilus (Malashenko et al., 1979), Methyloccocus sp (MN-222) and an unidentified methanotroph H-2 (Imai et al., 1986). The nature of the inactivation and reactivation of the two groups of methanotrophs (thermotolerant and mesophilic methanotrophs) was investigated and then a suitable strain for the production of PO was selected.

10.2.2 Experimental

Each organism has a different optimal growth-, reaction- and reactivation temperature. However, for experimental purposes mesophilic methanotrophs were cultivated at 30°C and thermotolerant methanotrophs were grown at 45°C. The productivity of PO, inactivation and reactivation rates were mainly studied at the temperatures of 30, 35, 40, 45°C. Whole cell activity was measured at 45°C for all the organisms. The inactivation and reactivation were carried out as mentioned in Materials and Methods.

10.2.3 Results

10.2.3.1 The Reactivation Patterns of Thermotolerant Methanotrophs

Nine thermotolerant strains including Methylococcus capsulatus (Bath) were investigated. The reactivation of cells, which were inactivated by acetylene and also following the production of PO, are shown in Fig. 10.2.1 and Fig. 10.2.2 respectively. All the strains tested showed reactivation. The cells which were inactivated by acetylene were reactivated more than two times faster than that of propylene-inactivated cells. It was very difficult to compare the tolerance, stability or reactivation rates of the various strains because the potential cell activities before the reaction started and also the peak productivities were different for each strain. However, a similar relationship between peak productivity and the time taken for the cells to reactivate completely was observed among strains, as had been seen previously in Methylococcus capsulatus (Bath).

The loss of activity following the production of PO was similar in each organism, however four strains were obviously reactivated faster

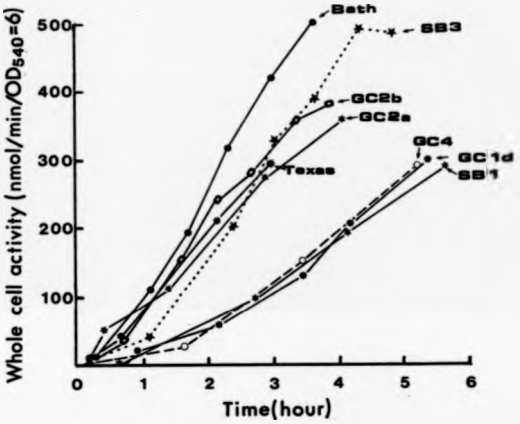


Fig. 10.2.1 Reactivation of acetylene-inactivated cells of thermotolerant methanotrophs

Methylococcus capsulatus Bath
M. capsulatus Texas

The others are not identified.

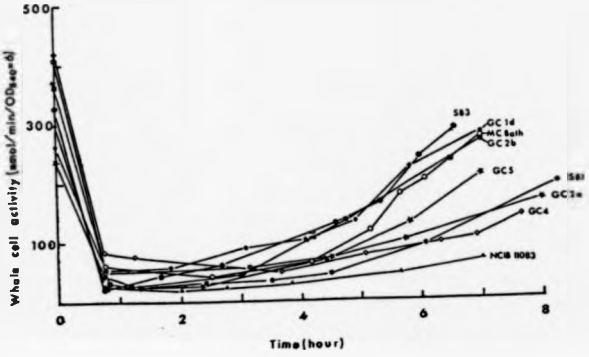


Fig. 10.2.2 Reactivation of propylene-inactivated cells of thermotolerant methanotrophs

Methylococcus capsulatus (Bath)

Methylococcus sp. NCIB 11083

The others are not identified.

than the others. These strains were SB-3, GC-1d, GC-2b and Methylococcus capsulatus (Bath). However a significantly superior strain compared with Methylococcus capsulatus (Bath) was not found. Accordingly, mesophilic methanotrophs which are disadvantageous for the recovery of PO (since they grow less than 40°C) than thermotolerant methanotrophs, were investigated.

10.2.3.2 The Reactivation Patterns of Mesophilic Methanotrophs

Reactivation patterns of cells which were inactivated by acetylene and following the production of PO are shown in Fig. 10.2.3 and Fig. 10.2.4 respectively. All the strains which were tested showed reactivation. The reactivation rates of acetylene-inactivated cells were also twice as fast as those which had been propylene-inactivated. The reactivation rates of propylene-inactivated cells were relatively high compared with the thermotolerant strains. This may be caused by the difference of the reaction- and reactivation-temperatures. The peak productivities of mesophilic organisms were found to be lower than that of the thermotolerant methanotrophs. These low productivities may enhance the reactivation rates of the mesophilic methanotrophs.

In mesophilic organisms, the Type II methanotrophs such as Methylocystis parvus (OBBP) or Methylosinus trichosporium (OB3b) were obviously inactivated less and also showed higher reactivation rates than Type I methanotrophs. The major differences between these two types of mesophilic methanotrophs lie in their internal membrane structure and their carbon assimilation pathways.

The reactivation rates of acetylene-inactivated cells was almost the same in thermotolerant- and mesophilic-strains. This indicated that the reactivation rates of MMO and rate of protein synthesis were similar. Nevertheless, the reactivation rates of propylene-inactivated

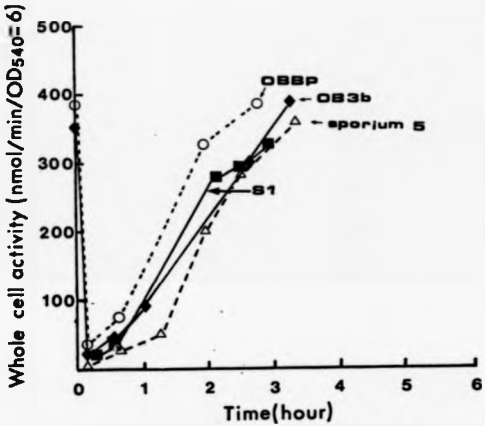


Fig. 10.2.3 Reactivation of acetylene-inactivated cells of mesophilic methanotrophs

■	Methylomonas	methanica	S1
○	Methylocystis	parvus	OBBP
◆	Methylosinus	trichosporium	OB3b
△	M.	sporium	5

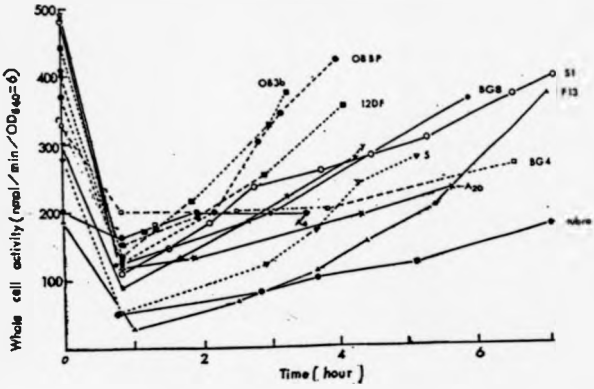


Fig. 10.2.4 Reactivation of propylene-inactivated cells of mesophilic methanotrophs

Strain			Type
Methylomonas	methanica	A4	I
M.	methanica	S1	I
M.	agile	A20	I
M.	rubra		I
M.	albus	BG8	I
M.	sp.	F13	I
Methylobacter	capsulate	Y	I
Methylocystis	parvus	OBBP	II
Methylosinus	trichosporium	OB3b	II
M.	sporium	12DF	II
M.	sporium	BG4	II
M.	sporium	5	II

cells in thermotolerant strains were lower than those of mesophilic strains. Lower reactivation rates of thermotolerant strains might be caused by the difference between concealed inactivation and reactivation.

However, the reactivation system is a common property of methanotrophs and this mechanism must be important for survival in their natural environment. In next Section, three promising strains which are Methylococcus capsulatus (Bath), Methylocystis parvus (OBBP) and Methylosinus trichosporium (OB3b) are compared further.

10.3 Which Organism is Better as a Biocatalyst for the Production of PO?

Methylococcus capsulatus (Bath), Methylocystis parvus (OBBP) and Methylosinus trichosporium (OB3b) were the superior organisms within the thermotolerant and mesophilic strains for PO production. However these three organisms have different optimal temperatures for cell growth, reaction and reactivation. In order to compare these organisms, inactivation and reactivation patterns were investigated under various temperature regimes. The results are shown in Fig. 10.3.1.

The inactivation and reactivation rates of Methylocystis parvus (OBBP) and Methylosinus trichosporium (OB3b) at 35°C were better than that of Methylococcus capsulatus (Bath) at 45°C, but the peak productivity of Methylosystis parvus (OBBP) and Methylosinus trichosporium (OB3b) were less than 65% of that of Methylococcus capsulatus (Bath). To compare the organisms under similar conditions, the reaction temperature of Methylococcus capsulatus (Bath) was decreased from 45°C to 40, 35 and 30°C. The results are shown in Fig. 10.3.2.

By decreasing the reaction temperature, the peak productivity was depressed compared with the reaction at 45°C and the inactivation rate was also decreased, furthermore the reactivation rate was increased.

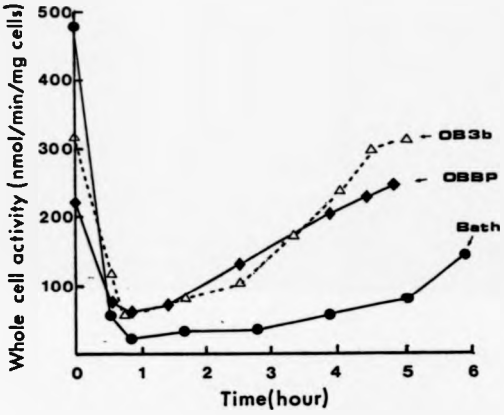


Fig. 10.3.1 The effect of reaction temperatures on the inactivation and reactivation of cells

		Reaction temperature	Peak productivity	Reactivation temperature
●	M.c Bath	45 °C	310 mU	45 °C
△	M.t OB3b	35	205	35
◆	M.p OBBP	35	195	35

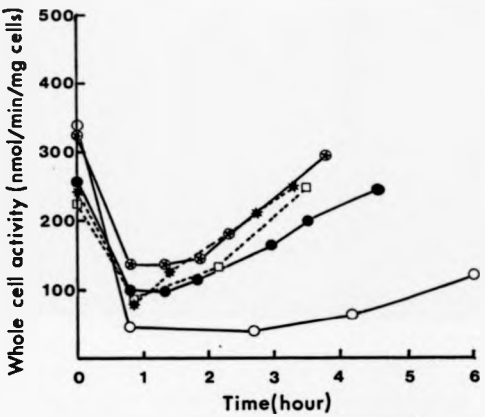


Fig. 10.3.2 The effect of reaction temperatures on the inactivation and reactivation of cells in Methylococcus capsulatus (Bath)

		Reaction temperature	Peak productivity	Reactivation temperature
●	M.c Bath	30 ℃	30 mU	45 ℃
●	"	35	145	45
○	"	40	180	45
✱	M.t OB3b	30	180	30
□	M.P OBBP	30	185	30

Under the same reaction temperature (30°C), Methylocystis parvus (OBBP) and Methylosinus trichosporium (OB3b) showed less inactivation rates and higher reactivation rates than Methylococcus capsulatus (Bath). Typical properties of the three strains are listed in Table 10.3.1. If a method of PO separation was available which is similar at 35°C and 45°C, Methylocystis parvus (OBBP) would appear to be the most suitable organism for PO production. At temperatures below 40°C the volatility of PO is low and may therefore cause inhibitory problems due to its retention within the bioreactor. Isolation of new thermotolerant or thermophilic strains could be valuable in the development of a viable PO process in which PO solubility would be low and therefore less inhibitory.

In the next section, the stoichiometry of the methanol consumption and PO production which is one of the most important parameters on economics of the process is discussed.

10.4 The Stoichiometry of the PO Production and Methanol Consumption

The stoichiometry of PO production and methanol consumption (PO/methanol) is one of the most important economic factors for the production of PO. In the resting cell reactions, methanol is used for the oxidation of propylene. The amount of energy required for the production of PO can be calculated from the stoichiometry of PO/methanol (mol/mol) using a shake flask assay.

The stoichiometries were 1.2 and 0.8 in Methylococcus capsulatus (Bath) and Methylocystis parvus (OBBP) respectively (Table 10.4.1). The amounts are lower than calculated theoretically from the catabolic pathway or had been reported by Subramanian (1986), Leak and Dalton (1983). If formaldehyde dehydrogenase and formate dehydrogenase are linked with NADH and coupled with NMO, the stoichiometry of PO/methanol

Table 10.3.1

The typical properties of the three methane-oxidising bacteria, Methylococcus capsulatus (Bath), Methylocystis parvus (OBBP) and Methylosinus trichosporium (OB3b)

Property	M. capsulatus (Bath)	M. parvus (OBBP)	M. trichosporium (OB3b)
Cell growth (μ, hr^{-1})	0.2 - 0.25	0.15 - 0.2	0.15 - 0.2
High biomass culture (g/l)	10	30	15
Whole cell activity (mU/mg cells)	500 - 700	250 - 350	250 - 350
Reaction temperature (°C)	45	35	35
PO/methanol stoichiometry (mol/mol)	1.2	0.8	1.0
Reactivation rate (mU/min)	< 2	< 2	< 2

Table 10.4.1

The stoichiometry of the methanol consumption and PO production

Strain	Methanol added[a] (μmol)	PO produced (μmol/mg cells) total[b]	endogenous[c]	Stoichiometry (mol/mol)
M. parvus (OBBP)	1.0	0.962	0.124	0.838
	1.5	1.402	0.183	0.812
	2.0	1.432	0.226	0.603
M. capsulatus (Bath)	1.0	1.286	0.082	1.204
	1.5	1.734	0.132	1.068
	2.0	2.362	0.154	1.086

Stoichiometry (PO/methanol) = $\frac{b - c}{a}$

must be more than 2.0. This is because methanol dehydrogenase can also supply energy (see Chapter 3).

The stoichiometry of PO/methanol was affected by the initial concentration of methanol. When 1mM methanol was used, the stoichiometries were high although by increasing the methanol concentration, the efficiency of methanol as an electron donor was decreased, both in Methylocystis parvus (OBBP) and Methylococcus capsulatus (Bath). A similar phenomenon was also observed when n-butanol was used as an electron donor (by coupling with methanol dehydrogenase and particulate MMO, Fig. 10.4.1). Usually the methanol dehydrogenase activity was two times higher than the MMO activity in Methylocystis parvus and Methylococcus capsulatus (Bath) (See Chapter 3, 9). Hence, when a sufficient amount of methanol for the methanol dehydrogenase was supplied, methanol dehydrogenase oxidised methanol at a maximum rate and provided energy. However this excess energy appeared not to be accepted by MMO efficiently. Accordingly, by increasing the methanol concentration, the stoichiometry of PO/methanol decreased (i.e. lowered efficiency). Furthermore, by increasing the methanol concentration, a percentage of methanol oxidation by MMO must have increased (as well as increasing the competitive inhibition) and this oxidation of methanol by MMO causes a loss in available energy. Under propylene oxidising conditions, the oxidation rate of butanol (as the electron donor) was 730 nmol/min/mg cells, however under non-propylene oxidation conditions, the oxidation rate of butanol was decreased to 530 nmol/min/mg cells (Fig. 10.4.1). This difference seems to be due to the existence of propylene. Despite this 70% of the electrons produced by methanol dehydrogenase were easily consumed without supplying energy to MMO. Accordingly, in order to obtain the high stoichiometry of PO/methanol, the feed rate of methanol as the electron donor must be carefully controlled at a level of below 1mM. The low stoichiometry of

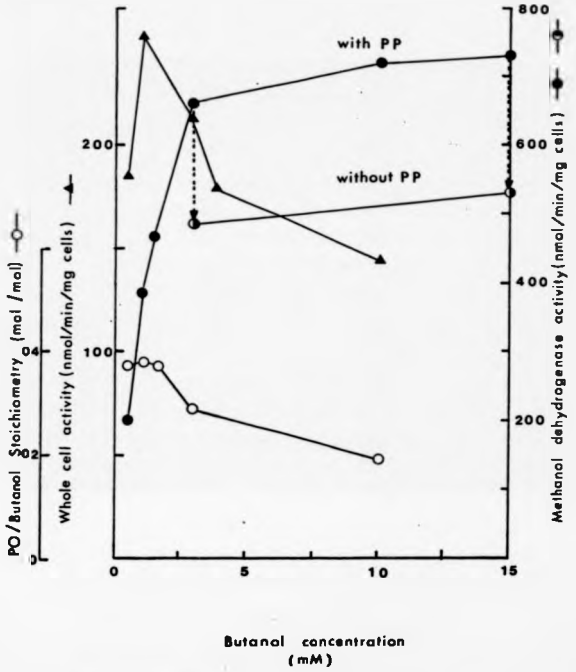


Fig. 10.4.1 The stoichiometry of the butanol consumption and PO production in Methylocystis parvus (OBBP)

PO/methanol increases the PO production cost.

In the next Section, the other important factors which are the consumption of carbon-, nitrogen- and sulphur-source in the reactivation process are discussed.

10.5 Substrates Consumption for Reactivation

10.5.1 Introduction

In the two stage reactivation process, carbon-, nitrogen- and sulphur sources (nutrients) are also required for the reactivation of inactivated cells. The need for these substrates, especially the carbon and nitrogen source increase the production cost of PO. To estimate the cost of nutrient consumption during reactivation, the nutrient consumption rates were investigated. Methylocystis parvus (OBBP) could be reactivated using endogenous carbon and nitrogen sources, so it was difficult to elucidate their overall nutrient consumption rates since both endogenous and exogenous nutrients could be immobilized. Methylococcus capsulatus (Bath) on the other hand was not reactivated without addition of exogenous nutrients. Hence, nutrient consumption rates were estimated using Methylococcus capsulatus (Bath).

10.5.2 Experimental

When the methanol consumption rate was examined, the cells were cultivated with methanol as a carbon source as described in Chapter 6. The cells were inactivated by the addition of acetylene or propylene as described in Materials and Methods. Then cells were washed twice with NMS medium and resuspended in the same medium. Methanol was then supplied continuously at a constant rate.

When nitrate- and sulphur consumption rates were examined, cells were cultivated with methane as the carbon source. Methane was also used as a carbon source for the reactivation of inactivated cells. The cells, inactivated by acetylene or propylene, were washed twice with NMS medium in which potassium nitrate was replaced with 0.5 g/l of potassium chloride when nitrate consumption rates were examined. Also magnesium sulphate in the NMS medium was replaced with 0.5 g/l of magnesium chloride when sulphate consumption rates were measured, but in this case, a small amount of sulphate was included in the trace elements solution. This amount was taken into account when the sulphate consumption rate was calculated. After washing with NMS medium, different amounts of potassium nitrate or magnesium sulphate were added at the beginning of reactivation treatment. The consumption rates (ideal consumption rates) were calculated as if all the nitrate or sulphate added were consumed completely at a constant rate at the time of the reactivation process.

10.5.3 The Necessary Amount of Carbon Source for the Reactivation

Cells, inactivated by acetylene, required methanol at a rate of about 50 nmol/min/mg cells (mU/mg cells) for the maximum reactivation rate (Fig. 10.5.1). No cell growth was observed under this condition. When the methanol addition rate was increased to more than 80 mU/mg cells, cell growth was observed and the reactivation rates were decreased. This rate of 50 mU/mg cells was both necessary and sufficient.

Cells, inactivated under the conditions of PO production, required a methanol addition rate of about 80 mU/mg cells (Fig. 10.5.2), increasing the methanol addition rate, increased cell growth as observed

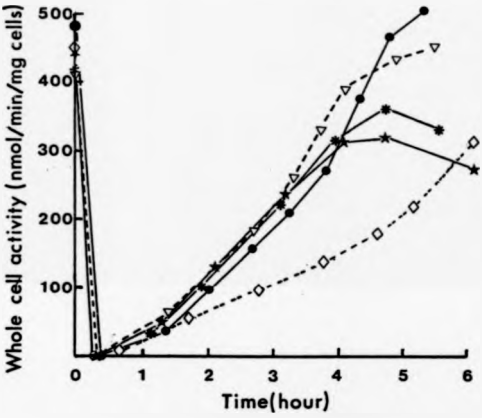


Fig. 10.5.1 The necessary amount of methanol for the reactivation of acetylene-inactivated cells in Methylococcus capsulatus (Bath)

Methanol feed rate (nmol/min/mg cells)	Cell growth
◇ 40	-
● 60	-
▽ 80	+
✱ 250	+++
★ 360	+++

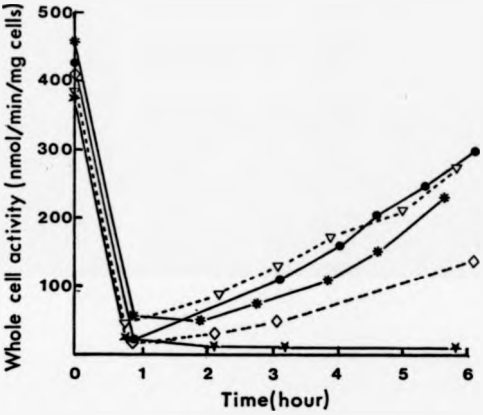


Fig. 10.5.2 The necessary amount of methanol for the reactivation of propylene-inactivated cells in Methylococcus capsulatus (Bath)

Methanol feed rate (nmol/min/mg cells)	Cell growth
◇ 60	-
✱ 75	-
● 90	-
▽ 110	+
★ 250	-

with the reactivation of acetylene-inactivated cells. When methanol feed rate was high (250 mU/mg cells), reactivation was inhibited. The total amount of methanol required to reactivate the cells was extremely different between acetylene- and propylene-inactivated cells, as the period for complete reactivation was two or three fold longer in propylene-inactivated cells. This difference in reactivation must be responsible for the difference in inactivation. Decreasing the reactivation period by accelerating the reactivation rate is very important to conserve the carbon and energy source.

10.5.4 The Necessary Amount of Nitrogen Source for the Reactivation

The necessary and sufficient amount of nitrate for reactivation was about 0.5 g/l for the cell concentration of 1.67 g/l in acetylene-inactivated cells (Fig. 10.5.3). This amount can be calculated as about 15 nmol nitrate/min/mg cells (mU/mg cells) if all the nitrate added was consumed at a constant rate within 4 hours. When the potassium nitrate concentration was less than 0.5 g/l, the cells reactivated to a certain level once, then the cells lost their activity again. However during this period, the cells continued to grow. A slow reactivation rate was obtained when nitrate was not supplied (dinitrogen was present).

The nitrate consumption rate of propylene-inactivated cells was higher than that of acetylene-inactivated cells (Fig. 10.5.4). The necessary and sufficient amount of nitrate required for reactivation of propylene-inactivated cells calculated from experimental data to be about 35 mU/mg cells. This amount was twice that required for acetylene-inactivated cells. No reactivation was observed when dinitrogen was supplied as a possible nitrogen source.

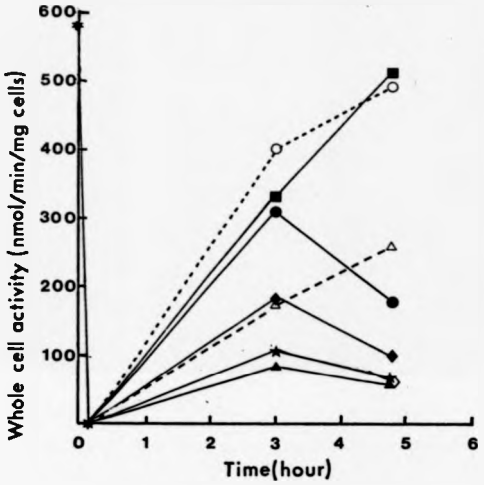


Fig. 10.5.3 The necessary amount of nitrate for the reactivation of acetylene-inactivated cells

Nitrate consumption rate (nmol/min/mg cells)

- ▲ 0
- ★ 1
- ◆ 5
- ● 10
- ○ 30
- ■ 100
- △ 300

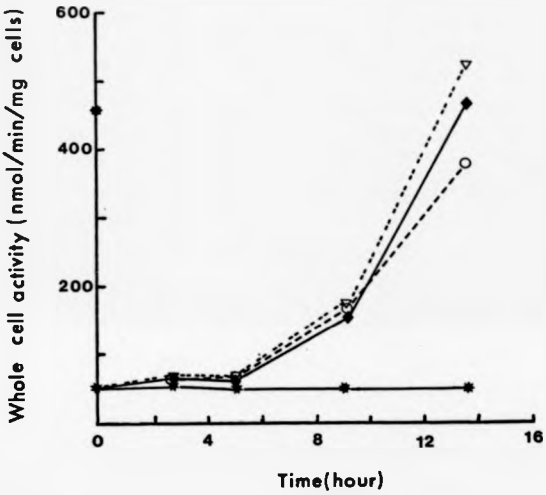


Fig. 10.5.4 The necessary amount of nitrate for the reactivation of propylene-inactivated cells

Nitrate consumption rate (nmol/min/mg cells)

- ✱ 0
- ○ 30
- ◆ 40
- ▽ 50

10.5.5 The Necessary Amount of Sulphur Source for the Reactivation

The necessary amount of sulphur source was also estimated using a similar method as for the nitrogen source consumption experiments. Magnesium sulphate was added at various concentrations to the acetylene-inactivated cell suspensions. The results are shown in Fig. 10.5.5. Under sulphate-limited conditions cells showed reactivation during the initial 3 hours after inactivation, but lost their activity after this period because they entered sulphur-limiting conditions. The necessary and sufficient amount of sulphate required was about 0.8 mU/mg cells. The propylene-inactivated cells showed the similar consumption rate (Fig. 5.5.6) and the necessary and sufficient amount of sulphate was about 0.8 mU/mg cells.

10.5.6 Discussion

The molar ratio of methanol, nitrate and sulphate to be present in the necessary and sufficient amount was 50 : 15 : 0.8 in acetylene-inactivated cells and 80 : 35 : 0.8 in propylene-inactivated cells. Propylene-inactivated cells require more nutrients for reactivation, suggesting that the damage to the cell occurs in other systems besides MMO. The phenomenon that cells lose their activity after they have recovered once when the nitrogen and/or sulphur source are limiting can be possibly explained as follows. The cells use the nitrogen source for cell replication preferentially over reactivation, if the carbon source is present in a sufficient or excess amount. However, in case of carbon-limiting conditions, this phenomenon cannot be observed because cells can not grow under this condition. When the carbon source is in excess, the decrease of reactivation which occurs indicates that reactivation and cell growth are competing for nutrients.

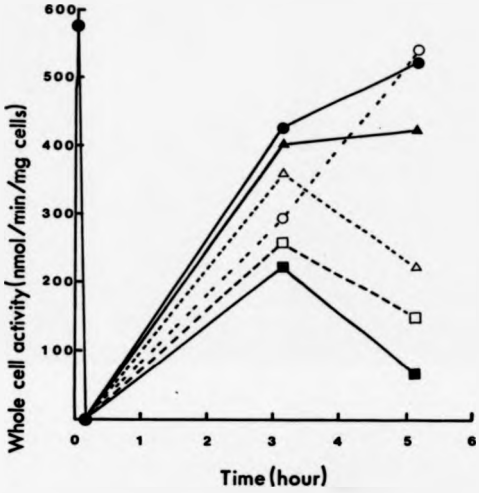


Fig. 10.5.5 The necessary amount of sulphate for the reactivation of acetylene-inactivated cells

Sulphate consumption rate (nmol/min/mg cells)

- ■ 0.02
- □ 0.12
- △ 0.22
- ▲ 0.52
- ● 1.0
- ○ 5.0

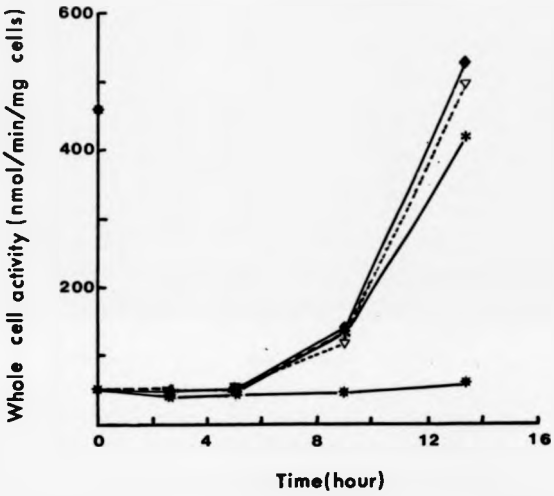


Fig. 10.5.6 The necessary amount of sulphate for the reactivation of propylene-inactivated cells

Sulphate consumption rate (nmol/min/mg cells)

- ✱ 0.04
- ✱ 0.44
- ◆ 0.84
- ▽ 1.84

As presented in Chapter 9, an excess of nitrate causes the accumulation of nitrite. However, the conditions of limiting nitrogen source depresses the reactivation. Moreover, excess carbon source also depresses the reactivation. Therefore the supply of nutrients to the reactivator needs to be carefully controlled.

The consumption of carbon and nitrogen sources during reactivation increases the cost of PO production. By increasing the reactivation time, the total amount of methanol required for production of PO is also increased. If it takes more than 20 hours for complete reactivation then the reactivation system becomes less attractive compared to the use of cells once, when the carbon conversion efficiency for cell growth is 0.3.

The supply of nutrients in the reactivation system may be a reflection of the maintenance energy requirement. The concept of maintenance energy was first proposed by Pirt et al. (1975). Briefly it suggests that cells need to divert a proportion of their metabolism into 'housekeeping' functions such as repair and maintenance of structural macromolecules within the cells. The maintenance energy has been calculated for Zymomonas mobilis during ethanol production by Tempest (1978), Pieschko and Humphrey (1983) and Beyeler et al. (1984). These groups showed that the maintenance energy rate to be 1.46 - 2.23 g glucose/g cells/hr. Pieschko and Humphrey (1983) reported a value of 2.13 g/g at an ethanol concentration of 27 g/l, however when an average ethanol concentration of 9.2 g/l/hr the value of the maintenance energy declined to 0.55g/g/hr. This is because at lower ethanol concentrations less damage occurs in the cells therefore less energy is required for maintenance.

In the case of propylene-inactivated cells of Methylococcus capsulatus (Bath), the methanol consumption rate to reactivate the cells was about 80 mmol/min/mg cells. This value can be expressed as 0.15 g

methanol/g cells/hr maintenance energy. The molecular weight of methanol is only one fifth of that of glucose. However, 0.15 g methanol/g cells/hr appears to be not so high compared to that of Zymomonas mobilis in ethanol production.

Having studied the parameters needed to evaluate the economics the next section will consider process economics for the biological processes.

10.6 Process Economics

As mentioned in the introduction, several biological PO production processes have been proposed. However, most of the processes were based on the experimental results obtained from very small scale operations in the laboratory, only the Catus Process had been developed to a pilot scale (1.000 l). As an introduction of this section, the economics of the Catus Process (Neidleman, 1981a,b, Geigert et al. 1983 a,b) will be discussed. Then the biological processes using methane-oxidising bacteria and chemical processes will be discussed.

10.6.1 The Biological Chlorohydrin Process - Catus Process

The Catus process consisted of three separate enzyme-catalyzed or chemical steps, some of which could be conducted simultaneously.

Step 1 Production of hydrogen peroxide, catalyzed by glucose-2-oxidase from D-glucose to D-arabino-2-hexosulose.

$$\underset{\text{D-glucose}}{C_6H_{12}O_6} + O_2 \xrightarrow[\text{glucose-2-oxidase}]{} C_6H_{10}O_6 + H_2O_2$$

Step 2 The hydrogen peroxide produced in Step 1 was used in an enzyme-catalyzed reaction of propylene in the presence of a halide ion to form a halohydrin, which can then be converted to an epoxide either chemically or by enzymatic catalysis.

$$C_3H_6 + KBr + H_2O_2 \longrightarrow CH_3CH(OH)CH_2Br + KOH$$

$$CH_3CH(OH)CH_2Br + KOH \longrightarrow C_3H_6O + KBr + H_2O \text{ (PO)}$$

Step 3 Finally, the D-arabano-2-hexosulose from the first step was separately hydrogenated by chemical means to D-fructose.

$$C_6H_{10}O_6 + H_2 \xrightarrow[\text{chemically}]{} C_6H_{12}O_6$$

D-fructose

The overall reaction for the production of propylene oxide is shown below.

Overall:

$$C_3H_6 + O_2 + H_2 + \text{glucose} \longrightarrow \text{fructose} + C_3H_6O + H_2O$$

(PO)

The overall process was basically the same as the chemical chlorohydrin process. Theoretically, this process seems appealing because only propylene and a small amount of hydrogen are consumed, and because fructose, an end product, has a higher value than glucose. Recently the fructose market has been expanding as a result of fructose being used as a sweetener for soft drinks. Apart from these advantages, the very low productivity and the product concentration were serious problems. According to Maidlemann (1981a,b) propylene bromohydrin was produced in a concentration of 0.1 - 0.3 mg/ml. Even neglecting the additional water added for the last step of saponification, the PO concentration would only be 0.02%. If the productivity had not been improved, more than 40,000 tonnes of halogenation reactor would have been necessary for the production of 30,000 tonnes of PO per year

assuming 300 operational days. The PO productivity of this process was only 2.4 g/l/day. Furthermore, recovering PO from such a diluted state would be prohibitively expensive and the fructose would also have to be purified for food use. This process was highly dependent on the high-value by-product D-fructose, however by-product prices fluctuate considerably. For example, the development of artificial sweetener such as aspartame or over production of sugar may alter the market price of D-fructose. It is easy to presume that the Cetus Process is economically non-feasible, without any details of production costs. Recently Kawakami et al. (1986) have demonstrated the further development of this process. They improved the productivity of PO, however it is in the early stages of laboratory research and cannot be adequately evaluated here.

10.6.2 The Production of PO using the Growing-Cells Process

10.6.2.1 Introduction

There are two ways in which whole cells can be used as biocatalysts: either as living cells or as dead cells. The living cell method can be subdivided into two classes, a) the resting-cell method and b) the growing cell method. When the biocatalyst is stable, the dead cell method or resting-cell method is of greater merit than using the growing-cell method for product selectivity or product-recovery. This is because under growing-cell conditions unfavourable by-products are often produced. Moreover components in the medium might obstruct the purification of the product. Thus, either dead-cell or the resting-cell methods are applied in the production process of L-amino acids and organic acids (see Chibata and Tosa, 1984) using biotransformations.

On the other hand, the growing-cell methods have been used when the

biocatalysts have proved to be rather unstable. The traditional fermentation processes are included in this broader concept. The production of ethanol, acetic acid using immobilized growing cells are typical examples of this technology.

PO production using the growing-cell process is considered to be more practical than the resting-cell process when a multiple step enzymatic reaction is required for producing a compound. By keeping the cells under growing-cell conditions, cells can maintain the activities of any enzymes critical for the overall reaction. No report has been demonstrated on the production of PO using the condition of growing-cell in methanotrophs. By using the mechanism of reactivation, PO production under the conditions of growing-cell was undertaken. If the reaction and the reactivation could be achieved simultaneously, this would greatly reduce the capital cost of the system since no separate reactivator would be required.

10.6.2.2 Experimental

The cells of Methylocystis parvus (OBBP) were cultivated using methane as the carbon and energy source at 35°C in a 700 ml fermenter. The components of the NMS medium in which potassium nitrate was removed were doubled when the OD_{540} reached a value of 28 (7 g/l) to avoid any limitation by medium component. When the biomass concentration reached OD_{540} values of 56 and 84, the medium components were also increased by 3 and 4 times respectively. Copper concentrations were also changed from 3 mg $Cu\,SO_4.5H_2O$/l at an initial concentration to 6, 9 and 12 mg/l respectively. Agitation speed and pH were kept constant at 1000 r.p.m. and 7.2 respectively. 0.5M nitric acid was used to control pH.

The reaction was initiated by feeding propylene to the fermenter. Methane was used as the electron donor and carbon and energy source for

growth and reactivation. The gas composition and flow rates were varied so as to remove the PO and keep the supply of propylene, oxygen and methane sufficient to maintain cell-growth and allow reactivation to occur.

10.6.2.3 PO Production under Growing Cell Conditions using Methylocystis parvus (OBBP)

Before we introduce the results of the production of PO under the growing-cell condition, a few terms are defined again:

a) Specific cell activity: the activity of cells (nmol/min/mg cells = mU/mg cells).

b) Specific PO productivity: the production rate of PO per unit cells per unit time (nmol/min/mg cells = mU/mg cells).

c) Net PO productivity: the production rate of PO per unit volume of reactor per unit time (g/l/day).

In order for cell growth to occur under conditions of PO production, the cells must exhibit some tolerance for PO. Three strains, Methylocystis parvus (OBBP) and Methylosinus sporium (BOA) and Methylosinus trichosporium (OB3b) all produced PO continuously under growing-cell conditions. Methylocystis parvus (OBBP) was used for the further experiments. The results are shown in Fig. 10.6.1, Fig. 10.6.2. For both the low biomass (Fig. 10.6.1) and the high biomass concentrations (Fig. 10.6.2), it was necessary to keep the PO concentration below 1.3 mM for cell growth to occur and to keep the cell activity constant. The specific PO productivity and the specific cell

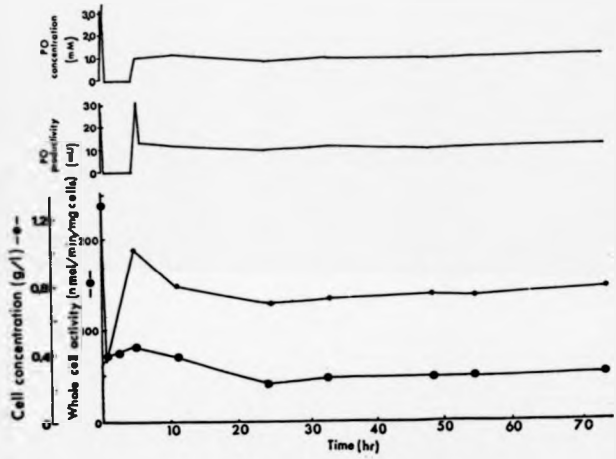


Fig. 10.6.1 PO production under growing cell condition using Methylocystis parvus (OBBP) - low biomass condition -

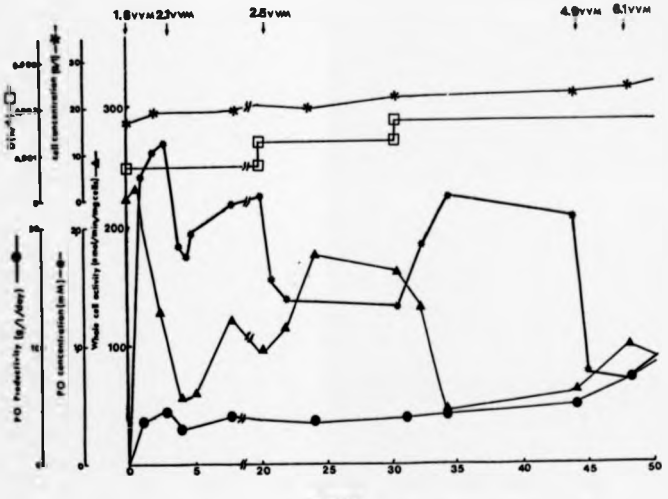


Fig. 10.6.2 PO production under growing cell condition using Methylocystis parvus (OBBP) - high biomass condition

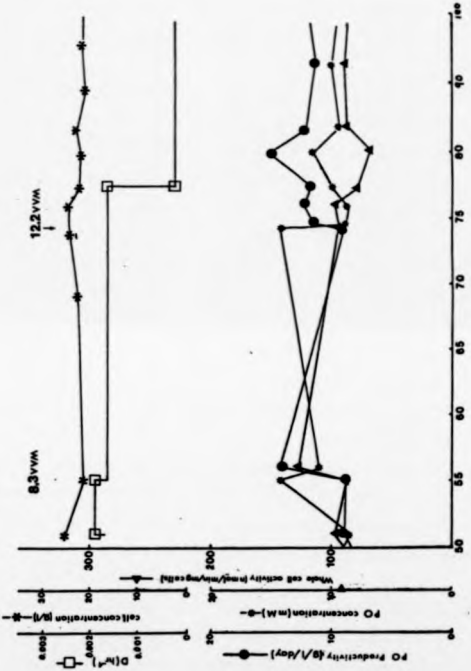


Fig.10.6.2 (continued)

activity were always in an inverse relation and the PO concentration and the specific cell activity were also in a reverse ratio. By the increase of specific PO productivity, the specific cell activity was decreased. The specific PO productivity in each experiment was 6 - 10 mU/mg cells. This specific PO productivity means that only 0.8 g PO produced/l/day (biomass: 1g/l) of net PO productivity or less would be produced.

To increase the net PO productivity, the biomass was increased gradually by decreasing the dilution rate and increasing the concentration of nutrients in the medium (Fig. 10.6.2). Gas flow rates were also increased to keep the PO concentration below 1.3mM in the culture. By increasing the biomass (to more than 15 g/l), the specific PO productivity was not increased (8 - 11 mU/mg cell), but the net productivity was increased and reached 12 g/l/day. No further increase in net productivity was seen when the biomass was increased to 24 g/l. This suggested that some factor other than cell concentration was limiting the net productivity. To investigate the limiting factor for the production of PO, methanol was supplied continuously to the reactor. The net productivity increased to 15 g/l/day after 2 hours then gradually decreased back to 12 g/l/day. This phenomenon can be explained if we assume that the electron donor (methanol) is the limiting factor. Alternatively it could mean that the reactivation rate is insufficient to maintain a productivity of 15 g/l/day.

If it is possible to keep the PO concentration in the reactor low a higher productivity may be obtained. However, the gas flow rate in the reactor had already reached 12 VVM, so from an engineering point of view, a different method should be used to remove PO.

The specific PO productivity was similar to the values which have been demonstrated using Nocardia corallina (B276) or Mycobacterium (see Chapter 3), but the net productivity of 12 - 15 g PO/l/day, obtained in

a continuous system, was the highest recorded value for PO production. The above productivity is at least 4 times higher than that the results reported in the short period experiments by Furuhashi et al. (1981), Habets-Crutzen et al. (1984) and Miyazaki et al. (1986). The growing cell PO production could be continued for more than 3 weeks. In the next Section, economics of the growing-cell process is discussed.

10.6.2.4 Process Economics of Growing-Cell Process

As shown in the last section, Methylocystis parvus (OBBP) was able to produce PO under conditions of cell growth. In the growing-cell reaction, cells inactivated by both exogenous and endogenous PO must be reactivated simultaneously. If the volumetric productivity can be increased by increasing the biomass, then the growing-cell reaction would be much more economically attractive, in capital cost terms, than a two stage reactivation process. For example, a volumetric productivity of 15 g/l/day in a single stage reactor would be equivalent to a value of 60 g/l/day in a two stage reactor process with a volume ratio of reactor/reactivator of 1:3.

The conceptualized process flow-sheet of the growing-cell process is presented in Fig. 10.6.3. The characteristic of this process is that cell replication, PO production and reactivation occur simultaneously in the same vessel. At the outset, sterile medium is fed into the reactor (and also the reactivator). The seed culture is then transferred to the reactor and the cells are grown as a batch culture by supplying a methane and air gas mixture. Carbon dioxide is thus generated by the cell growth. In order to recover methane, the exhaust gas is recycled by passing it through the hot potassium carbonate absorber (117°C) to absorb the CO_2 under high pressure conditions (20 bar). When the biomass reaches a fairly high level propylene is fed through the sterile

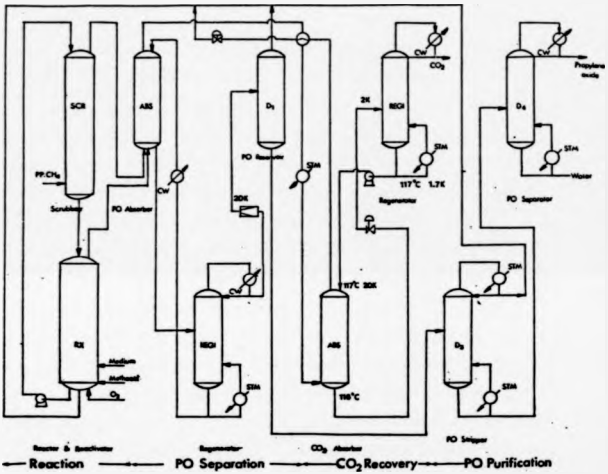


Fig. 10.6.3 The conceptualized process flow-sheet of the growing-cell process

filter to the reactor. Using automatic gas analysis, fresh oxygen and propylene are appropriately supplied to the reactor. Methane is also supplied as an electron donor and to maintain cell growth. If the supply of electron donor is insufficient then extra methanol can be added as required.

PO is thus produced rapidly under high biomass conditions and accumulates in the reactor. To keep the PO concentration in the reactor low, a scrubber is attached to the top of the reactor. The reaction mixture is then pumped up from the reactor to the scrubber and recycled to reactor. Propylene is used as a stripping gas in the scrubber so as to avoid unnecessary inactivation during scrubbing. A partial vacuum is maintained in the scrubber to remove the PO and to increase the PO concentration in the effluent gas. Gas leaving the scrubber is first of all passed through the PO-absorber which is filled which absorbent to concentrate the PO. Low boiling point liquid-hydrocarbons, fluorocarbons, ethers or alcohols can be used as absorbers. The out-going gas from the PO-absorber is then transferred to the CO_2 absorber as mentioned before. The out-going gas from CO_2-absorber is finally directed to the reactor. CO_2 is released from the hot potassium solution by decreasing the pressure from 20 to 1.7 bar.

The PO trapped in the absorber is released by increasing the temperature to 60°C. The vapourized gas, which contains PO, propylene, water and small amounts of oxygen, nitrogen and CO_2 is then compressed so as to condense the gaseous PO to a liquid. The compressed gas is cooled and transferred to the liquid-gas separator. This liquid which contains PO, propylene, water and traces of oxygen, nitrogen and CO_2 is then transferred to the PO purification chamber. The liquid produced is fed into the regenerator for the removal of volatile gases such as

propylene and, oxygen. The liquid mixture remaining of PO and water is then transferred to the distiller in order to recover the pure PO product. Water is absolutely essential for the reflux of PO to purify.

Drozd (1986) noted that one gram (dry weight) of ethylene-utilizing bacteria catalyzed the production of only 0.1g of propylene oxide even when the PO was continuously stripped from the reactor. He also noted that this amount was extremely low compared with the production of 50g of ethanol from glucose using one gram (dry weight) of non-growing yeast cells.

The maximum productivity obtained by the growing-cells of Methylocystis parvus (OBBP) reported in this thesis was 15 g/l/day under the conditions of 20g dry cell weight/l biomass at a dilution rate of 0.0025 hr^{-1}. In this particular case, one gram of cells were capable of producing 12 g/l propylene oxide throughout their active life in the system (calculated from dilution rate and its mean retention time in the system was 16 days). This yield is significantly higher than previously recorded values (Hou, 1984b; Furuhashi et al, 1981). However this value is still a quarter of the rate of ethanol production of yeast. Therefore a further increase in PO productivity is necessary if an economically viable process is to be devised. If this productivity of 15 g/l/day is not improved, 8,000 tonnes of reactor would have been necessary for the production of 30,000 tonnes of PO per year assuming 300 operational days. The capital cost is too expensive.

In the next Section a more detailed cost analysis will be considered using the two-stage reactivation process.

10.6.3 Process Flow Sheet of the Two-Stage Reactivation Process

At the beginning of this project it was hoped that the problems of the product-toxicity and short life-span would be overcome as a result

of further advances in technology. However, at present it is difficult to prolong the life-span of the biocatalyst without using a reactivation step. A new bioprocess "The two-stage reactivation process" was designed to enable an accurate calculation of the production cost to be made. This process was designed based on the results of the continuous PO production and reactivation study in this project.

The conceptualized process flow-sheet is illustrated in Fig. 10.6.4. Cells cultivated in the seed-tank are transferred to the reactivator. This provides the cells for the reactor. Propylene, oxygen and methanol are fed into the reactor. In order to increase the oxygen supply to the cells, the reactor-pressure can be set at 3 bar. The reactivated cells are supplied continuously from the reactivator in order to ensure that the cell activity in the reactor remain constant. The effluent gas is then transported to the large PO absorber to remove PO from gas phase. To keep the PO concentration in the reactor low (below 3mM), reaction mixture is passed through the scrubber placed on the top of the reactor. This effluent gas is also transported to the PO absorber.

The partially inactivated cell suspensions in the reactor is pumped out into the vaccum scrubber to completely remove the PO. The PO, stripped out in the vacuum scrubber, is compressed and then transferred to the PO absorber. The cell suspension is transported to the reactivator. In the reactivator, cells are reactivated and grown simultaneously by supplying methane and the other nutrients.

The effluent gas from the PO absorber is compressed to remove the CO_2 produced by the oxidation of methanol in the reactor. The compressed gas is washed with a hot carbonate gas washer. The CO_2 absorbed by the carbonate solution can be removed by reducing the pressure in the liquid from 20 bar to 1.7 bar. After these, the gas mixture which contains propylene, nitrogen and oxygen is returned to the

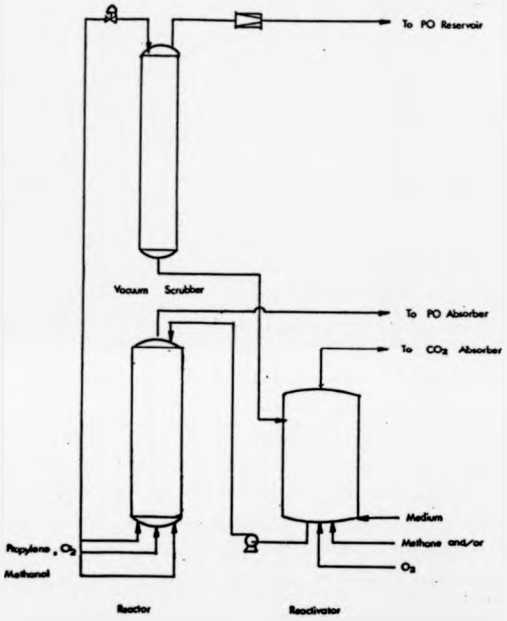


Fig. 10.6.4 The conceptualized process flow-sheet of the two-stage reactivation process

reactor. The recovery of the PO from the PO-absorber and the purification of PO are carried out by the same process as mentioned in the growing-cell process.

10.6.4 The Economics of PO Production using the Two-stage Reactivation Process

Before calculating the production cost of the two-stage reactivation process, the basic process parameters were set. These parameters were based upon both experimental and hypothetical results and are shown in Table 10.6.1. Methane consumption rate is assumed from the batch experiment (see Section 10.5.3) based on the methanol consumption rate. The time required for reactivating cell-activity is extremely important from the point of view of capital investment and energy (methane) costs. The shorter the reactivation time the cheaper the overall cost for the process. The average time proposed is 2 hours in the case of Methylocystis parvus (OBBP). The biomass concentration is capable of being maintained without any further processing of concentration at a level of 30g dry weight/l as presented in the growing cell reactor. The temperature of both the reactor and scrubber have a considerable effect on the removal of PO, consequently, at low temperatures the gas flow rate and volume of the PO absorber are increased to compensate. The specific PO productivity obtained using the continuous two-stage reactivation system in this project is 150 mU/mg cells. However, the above level of productivity can only be obtained under such conditions where the retention time of reactivator is kept below 15 minutes. As far as the specific PO productivity is concerned, this is postulated to be 80 mU/mg cells when the retention time is 1 hour in the reactor. The net productivity is also posturated to be 200 g/l/day from calculation based on 30 g/l biomass and a

Table 10.6.1

The basical parameters for the calculation of

PO production cost in two-stage reactivation process

(1) Cell growth and reactivation (Reactivator)	
Temperature	35°C
Biomass concentration at steady state	30g dry cells/l
Dilution Rate	$0.0025hr^{-1}$
Carbon source	methanol
Methanol consumption rate for the reactivation	80nmol/min/mg cells
Whole cell activity	130nmol/min/mg cells
Retention time of biocatalyst	2 hours
(2) Propylene oxide production (Reactor)	
Temperature	35°C
Biomass concentration	30g dry cells/l
Whole cell activity	90nmol/min/mg cells
Specific PO productivity	80nmol/min/mg cells
Net PO productivity	200g/l/day
PO/methanol in reactor	1.2 mol/mol
Retention time of biocatalyst	1 hour
Yield of PO	100%
PO concentration of out-going gas	0.1%

retention time ratio in the reactor and reactivator of 1:2 (1 hour:2hours) when specific PO productivity is 80mU/mg cells. A net productivity of 200 g/l/day was judged to be feasible if the half life of cells was more than 5 days when single stage reactor (resting cell, non-reactivation process) was used. Accordingly, the net productivity of 200g/l/day cells is thought to be a reasonable value both from experimental and economical points of view.

The PO concentration in the out-going gas which is mixed with exhaust from the reactor and scrubber is dependent on the conditions of operation. The higher the PO concentration in reaction mixture the higher the PO concentration of the gas phase. The stoichiometry of PO: methanol ratio is a factor which strongly influences the cost of the process. The PO:methanol ratio is 0.8 in Methylocystis parvus (OBBP), however the cells have a high endogenous reserves equivalent to 20 - 40 mU PO/mg cells. This is accumulated (possibly as PHB or poly-glucose) during the reactivation process. Accordingly, the PO:methanol ratio is now considered to be 1.2.

The PO production cost is shown in Table 10.6.2. At present an accurate calculation of the cost is difficult, because most of the parameters used in calculating the cost are based on the results of batch experiments or are assumed. For example, the net productivity in the reactor obtained in this project (Stanley, unpublished data) is 100 g/l/day (overall productivity taking account in reactivator is 15 g/l/day). This figure is a vast improvement over the published data. However, in this cost analysis, the figure of 200 g/l/day was used. This figure is arrived at by making calculations from such factors as specific productivity and biomass concentration. The ratio between the time in the reactor and the time in the reactivator is assumed from the results of batch experiment, however the ratio has not yet been achieved in any of our practical experiments (the best ratio of reactor/reactivator is 1/5).

Table 10.6.2

The PO production cost analysis in the two-stage reactivation process

	Production Overheads	Unit per kg	Price (£/kg)
RAW MATERIALS			
Propylene	0.519 £/kg	0.73	0.379
Methanol	0.170 £/kg	0.46 (reaction)	0.078
		1.10 (reactivation)	0.187
Total raw material			0.644
CATALYST			
Propylene glycol (as absorbent)	1.361 £/kg	(300 t/year loss)	0.014
Nutrients (mainly nitric acid)	0.255 £/kg	0.95	0.242
Total catalyst			0.256
UTILITIES			
Steam (300 psi)	12.718 £/tonne	4.8 tonnes/tonne	0.061
Electricity	0.063 £/kwh	5.32 kwh/tonne	0.034
Total Utility			0.095
VARIABLE COST			0.995
FIXED COST			
Inside battery limit (25%)		5.2 £ x 10^6	0.173
Outside limit (15%)		1.6 £ x 10^6	0.053
Total fixed cost			0.226
TOTAL COST OF PRODUCTION			£1.221/kg

Production capacity 30,000 tonne per year

Market price of propylene oxide £1.213/kg

The cost analysis (Table 10.6.3) contains the cost of towers and vessels and other equipment costs (pumps, heat exchangers, etc.) Prices are subject to alteration depending on the location of the plant. This cost analysis is based on the location in Japan. The fact that the production cost of 1.2 pound per kilogram is almost similar to the market-price suggests that even if the parameters which are proposed in the above calculations are achieved, this process of PO production is impractical. Generally speaking, the production cost of bulk chemicals is 60 - 80% of the market price because storage, transport costs and profit are required to be added to the production cost. Thus the market price using this biological route would be 1.5 - 2.0 pounds per kilogram. The reduction of energy and catalyst costs, especially the energy and nitrogen source for the reactivation, and capital costs (by decreasing the size of vessels) are the most important factors to consider in the future production of PO.

Table 10.6.3

The cost analysis of towers, vessels and other equipments

	Item	Price (£ K)
1	Reactors, Towers, Vessels	5,697
2	Heat exchangers	735
3	Pumps, Compressors	1,894
4	Electrical, Controllers, Pipes [(1 + 2 + 3) x 50%]	4,166
5	Construction [(1 + 2 + 3) x 100%]	8,328
	Inside battery limit	20,820
	Outside battery limit [Inside battery limit x 50%]	10,410

Production capacity 30,000 tonnes per year

Location Japan coast

CHAPTER 11

General Conclusions and Further Developments

Thirty years have passed since the biotransformation for the production of L-amino acids from N-acylamino acids was first used on a commercial basis. One of the main properties of a biotransformation, whether using enzymes or intact cells, is its optical selectivity. More than twenty biotransformation processes have been commercialized so far, and all of them, except the production of acrylamide, involve the production of optically active compounds. The production process for PO using methane-oxidising bacteria cannot, however, be included in the same category as the above processes. This is because PO produced by methanotrophs, is not optically active and its qualities are the same as those of chemically-manufactured PO. By effecting a decrease in the production-cost of PO to a level below that of existing processes is the only way for the biological process to be utilized commercially in the future.

An important property of MMO is its unusually broad substrate specificity. This property of MMO is commercially attractive because if a production process can be developed, it may be used in the manufacturing processes of many other compounds, using the same organism by a similar method. The development of a multi-purpose catalyst can lessen the research risk associated with the development of competititve technologies and market stability of product. Therefore, methane-oxidising bacteria are attractive biocatalysts for the production of PO and other oxigenated compounds.

Generally speaking, there are two types of catalyst: the disposable catalyst and the one which can be recycled. Biocatalysts belong to the former type, so the longevity of the catalyst is very important. When the life-span of the catalyst is short, its contribution to the production cost is high. Consequently the catalyst must be recovered and reused. However in the case of the biocatalyst used it was thought that the reactivation of an inactivated biocatalyst was very difficult.

Despite their many interesting properties, biocatalysts are unstable compared with chemical catalysts and hinder the development of biotransformation technology. The half life of the methane-oxidising bacteria was found to be less than 30 minutes under conditions of PO production. Even without the accumulation of external PO, the cells were inactivated immediately. When a fermenter was used for the production of PO, the inactivation rate of the cells was increased whilst the concentration of PO increased also. This phenomenon appears to have caused many scientists to conclude incorrectly, that the inactivation of the biocatalyst was due to the accumulation of PO, and the writer of this thesis was not an exception to this partial misunderstanding.

In an attempt to obtain insights into the real cause of inactivation it was necessary to separate the effects of externally-added PO on the biocatalyst from PO generated in vivo during biotransformation. Therefore, the adsorption of PO using adsorbents was studied at the outset to remove PO as it was termed. A novel adsorbent, "zeolite", which is made from silicate, and which is usually used as the support material of chemical catalysts was found to be effective for adsorbing epoxides selectively from the aqueous reaction mixture. The epoxides were easily recovered from the zeolite by soaking the latter in methanol solution. It was observed that zeolite did not appear to have any harmful effect on the cells. However, an adsorbent is unsatisfactory because it is only 70% efficient at extraction when used in the reaction mixture and this percentage of extraction efficiency remains constant even when the PO concentration in the reaction mixture is changed. Thus in the production of PO, this percentage is unsatisfactory to avoid the inactivation by the external PO.

The prevention of inactivation is the most important factor which requires the closest attention for the development of the biological PO

production process. Before studying those methods for the prevention of inactivation of cells, a clear explanation of the inactivation mechanism is necessary. To facilitate this the IBF-reactor was designed and provided a lot of information on the inactivation of cells i.e. the effect of the PO production rate on inactivation and the effect of external PO concentration on inactivation. However, inactivation mechanisms were not completely defined, although some inactivation mechanisms were assumed. They are as follows:-

a) The active site of MMO is inactivated by PO produced from propylene within the MMO active site.

b) The active site of MMO is inactivated by externally accumulated PO.

c) Extracellular and intracellular accumulation of PO inactivated formate dehydrogenase.

d) Methanol dehydrogenase was inactivated by PO, accumulated in cells under conditions of high PO production.

e) The concealed inactivation by PO which is accumulated within the cells during the oxidation of propylene.

As already mentioned, at least three enzymes were inactivated as a result of the production of PO. However, the inactivation mechanisms were thought to be more complicated. Therefore, combination of a variety of methods are necessary to prevent the inactivation of the biocatalyst. Firstly, in order to overcome the short life-span of the biocatalyst, reactivation of the inactivated cells was studied.

It was possible to reactivate a number of enzymes simultaneously

under the same conditions. It was found that the reactivation process could be repeated many times over, but unfortunately, the reactivation rate of the cells (which were inactivated under conditions of high PO production) was not as high as the rate with either acetylene-inactivated or PO-inactivated cells. The delay in the reactivation process was thought to be responsible for what has been called here "concealed inactivation." A mechanistic explaination for concealed inactivation has not been possible however, more detailed studies of the inactivation mechanism require to be undertaken. By using radio-labelled propylene or propylene oxide, an understanding into the mechanism of inactivation can be gained.

The tracer experiments will provide information concerning which proteins, or biological substances are alkylated by PO (or its matabolites). Such experiments could also provide information relating to what kind of materials are repaired during the reaction and the reactivation process. Some or all of the above topics may interest researchers in the future who find this field of work rewarding.

The inactivation rate of Methylocystis parvus (OBBP) was approximately 8 mU/min, when the specific PO productivity was 200 mU/min. However, the reactivation rate was only about 1 mU/min. Improvement of the reactivation rate by optimisation of the reactivation conditions, lead to a maximum reactivation rate of 2 mU/min. This calculation is assumed from the rate at which acetylene-inactivated cells are reactivated. The above estimate indicates that four times the reactivator volume, compared to the reactor volume is required. Furthermore, about twice the amount of energy is required for reactivation compared to that consumed for the oxidation of propylene in the reactor. Therefore, preventing the inactivation of cells is essential for cutting down the production cost.

Based on the information concerning inactivation mechanisms many

possible ways to prevent the inactivation were examined. This topic has not been fully developed in this thesis and has not been discussed. In this final Chapter, results obtained from experiments will be presented briefly below and certain important points will be discussed.

Compounds, such as amines or sulphide could mask the PO toxicity by reacting selectively with PO and/or its unstable derivatives. Moreover, there was a possibility that hydroxyl radicals might be involved in the inactivation of cells during PO production. More than forty compounds were tested to prevent inactivation by adding them to the reaction mixture of Methylococcus capsulatus (Bath) during PO production. Some of the results from the above experiments are shown in Fig. 11.1.1. None of them protected the inactivation of Mca under the conditions of PO production. However, some compounds such as benzoate, phenylacetic acid, tyrosine, methional etc. demonstrated acceleration of the reactivation process. These results suggested two possibilities. Firstly, one can conclude that partial prevention of concealed inactivation occurs and secondly, that there is an acceleration of reactivation by the compounds. The reasons for this acceleration of reactivation are not clear; nor is it obvious what it is that affects the acceleration per se. Most of the compounds which were able to show the above effect are known as radical scavengers. A typical radical scavenger, methional (Beauchamp and Fridovich, 1970) was decomposed by Methylococcus capsulatus (Bath) and Methylocystis parvus (OBBP). However, ethylene (or its MMO-oxidation product ethylene oxide) which is the end-product of the reaction with methional and hydroxyl radicals, was not detected.

In experiments not reported in this thesis, methional showed an acceleration of reactivation, but para-nitrosodimethylaniline (P-NDA), the other typical hydroxyl radical scavenger, did not. The latter compound is known to be bleached by hydroxyl radicals (Beauchamp and Fridovich, 1970). Actually, p-NDA was bleached by Methylococcus

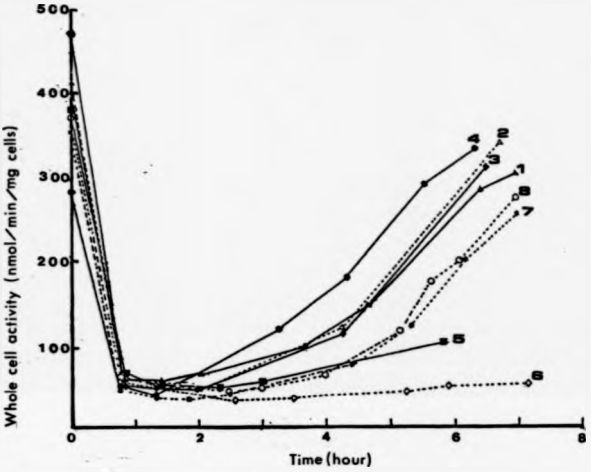


Fig. 11.1.1 The effect of additives on the prevention of inactivation and the acceleration of reactivation

Compound	Concentration	Peak productivity (mU/mg cells)
1 Benzoate	2.5 mM	270
2 Phenylacetate	0.875 mM	285
3 Tyrosine	22.5 mg/l	340
4 Methional	1 mM	250
5 Hydroxyurea	0.5 mM	215
6 Hydroxyamine	0.5 mM	205
7 Carbon dioxide	5 %	330
8 none (Control)		270

capsulatus (Bath) and Methyocystis parvus (OBBP) in the presence of oxygen and electron donor. The bleaching of p-NDA was independant of MMO activity because acetylene-inactivated cells also bleached p-NDA at the same rate as cells with high MMO activity. As shown above, all of hydroxyl radical scavengers did not show the protection effect, while some hydroxyl radical scavengers were found to be effective. The problem remains unsolved as to whether or not hydroxyl radicals are produced by methanotrophs, and if so whether or not hydroxyl radicals accelerate the inactivation of the biocatalyst.

One other interesting result was obtained. Carbon dioxide (and carbonates of its derivatives) and benzoate accelerated the productivity of PO. These compounds were also recognised as radical scavengers. The effect of sodium bicarbonate on PO productivity is shown in Fig. 11.1.2. PO productivities were increased in many methanotrophs tested, however proper explanations could not be found for this phenomenon. The manner in which those radical scavengers operate, especially with regard to their effect on methanotrophs requires extensive study.

The other method used to stabilize the biocatalyst is to treat it with chemicals in order to crosslink biopolymers in the cells. Polyethyleneimine and glutaraldehyde are often used for this purpose (see Chibata and Tosa, 1984). Methylocystis parvus (OBBP) was inactivated when their cells were treated with polyethyleneimine and they did not regain their activity. Melamine was selected as a low toxicity triamine to ligand with aldehydes. The cells which were treated with melamine at a concentration level of 0.2 g/l and formaldehyde appeared to be effective for preventing inactivation and also for accelerating reactivation (Fig. 11.1.3). These results indicate that there is a possibility of preventing inactivation of cells under the conditions of PO production and encourage research activity on the development of the PO production process.

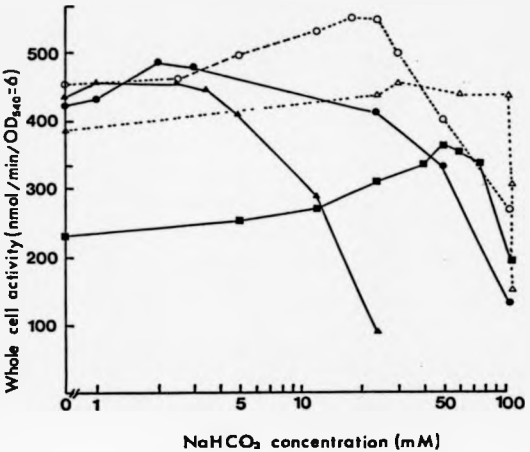
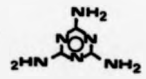


Fig. 11.1.2 The effect of sodium carbonate on the PO productivity in various methane-oxidising bacteria

△ Methylococcus capsulatus (Bath)
● Methylocystis parvus (OBBP)
▲ Methylosinus trichosporium (OB3b)
■ Methylosinus sporium (5)
○ Methylomonas albus (BG8)



melamine

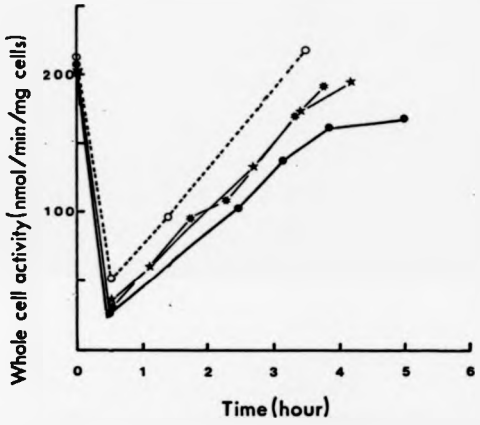


Fig. 11.1.3 The effect of melamine on the prevention of inactivation and the acceleration of reactivation

○ Melamine-formaldehyde treated cells
★ Melamine-glutaraldehyde treated cells
✱ Melamine-glyoxysal treated cells
● Control

In Chapter 8, it was assumed that a possible cause of the inactivation of cells derived from accumulation of intracellular PO. Hence a decrease in the intracellular PO concentration would appear to be the other important antidote. Not only would this overcome rapid inactivation, but would solve the problem of delayed reactivation. This thesis therefore concludes by recommending the prolongation of the short life-span of the cells, and increasing their reactivation rate.

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