APPLICATION OF A METHANOTROPHIC IMMOBILIZED SOIL BIOREACTOR TO TRICHLOROETHYLENE DEGRADATION

by

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A thesis submitted to the Department of Chemical Engineering

In conformity with the requirements for

the degree of Doctor of Philosophy

Queen's University

Kingston, Ontario, Canada

(Sep, 2008)

Abstract

Trichloroethylene (TCE) is a major groundwater contaminant and is a cause of serious health concern. Methanotrophic TCE degradation is very promising compared with other treatments. Methanotrophs produce methane monooxygenases (MMOs) which catalyze methane oxidation and cometabolize chlorinated and aromatic compounds. High rate of TCE degradation is attributed to only soluble MMO (sMMO) expressed mainly by type II methanotrophs under copper-deficient conditions. To make methanotrophic TCE degradation practical, high density methanotrophic biomass with high sMMO activity is required. Methane is the primary substrate for methanotrophs and sufficient quantities must be supplied to support biomass growth. Because of the poor water solubility of methane, mass transfer limitation essentially restricts high biomass production. When methanol was used as the growth substrate, biomass concentration of 7.4 g l^{-1} *Methylosinus trichosporium* OB3b was achieved in a 160-h fermentation using an exponential feeding strategy based on pre-determined μ. Even higher biomass density of 19 and 29 g l^{-1} biomass were obtained by a modified feeding strategy based on carbon dioxide production. It is concluded that methanol is a promising substrate for the production of large amounts of *M. trichosporium* OB3bbiomass. In addition, allylthiourea was applied to methanotrophs growth medium to circumvent the inhibitory effect of copper, which inhibits sMMO activity but not particulate MMO (pMMO). We successfully retained sMMO activity by supplementing allylthiourea. Even when *M. trichosporium* OB3b was grown with 4.5 μM copper, which would completely block sMMO expression, addition of 15 μM allylthiourea preserved half of the sMMO activity. It was also observed that switching the growth substrate

from methane to methanol did not significantly affect sMMO activity. An immobilized soil bioreactor was developed to examine the efficiency of methanotrophic TCE degradation by combining the knowledge obtained on high biomass production and applying allylthiourea for sMMO expression. In a batch TCE degradation experiment, about 63% of TCE was removed in 5.75 h. The maximal TCE degradation rate of 1.40 mg l^{-1} h^{-1} was obtained in a continuous TCE degradation at a dilution rate of 0.15 h^{-1}. This study demonstrated the effectiveness of a novel bioreactor system for methanotrophic TCE degradation.

Co-Authorship

The author wishes to acknowledge the contribution of Dr. Bruce A Ramsay and Dr. Juliana A. Ramsay in the supervision of and the preparation of all manuscripts presented in this thesis. All experiments were performed by the author.

Acknowledgements

I would like to thank all those people who made this thesis possible and an enjoyable experience for me.

I would like to express my sincere gratitude to Dr. Juliana Ramsay and Dr. Bruce Ramsay for their excellent guidance, constant encouragement, support, and invaluable suggestions. Without their help, this work could not be possible. Their advice and patience is appreciated. I would also like to thank Dr. Martin Guay for his supervision at the early stage of this research. I am very grateful to my committee members for their time and effort in reviewing this work.

I would like to acknowledge my labmates, Zhiyong Sun, Xuan Jiang, Bozhi Sun, Paul, and Femi, for providing valuable feedback and advice, and for sharing experiences and knowledge during the time of study. They have been great friends throughout my stay at Queens' University. The close cooperation with them is a great experience.

Finally, I am deeply and forever indebted to my parents for their love, support and encouragement throughout my entire life. I would like to express my deepest gratitude for the constant support, understanding, patience, and love that I received from my husband Wei Sun during the past years. One of the best experiences that we lived through in this period was the birth of our daughter Jasmine, who provided an additional and joyful dimension to our life.

Statement of Originality

(Required only for Division IV Ph.D.)

I hereby certify that all of the work described within this thesis is the original work of the author. Any published (or unpublished) ideas and/or techniques from the work of others are fully acknowledged in accordance with the standard referencing practices.

Yinghao Yu

September, 2008

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List of Abbreviations and Symbols

AOP	Advanced oxidation process
ATU	Allylthiourea
CAH	Chlorinated aliphatic hydrocarbon
CBC	Copper-binding compound
CCP_t	Cumulative carbon dioxide production, g
CPR	Carbon dioxide production rate, g h^{-1}
CSTR	Completely stirred tank reactor
D	Bubble diameter, m.
D_{gas}	Diffusion coefficient of gas in liquid phase, cm^2 s^{-1}
DCE	Dichloroethene
EPA	Environmental Protection Agency
FBR	Fluidized bed reactor
H_{CH4}	Henry's law constant for CH_4 , atm mg^{-1} l^{-1}
ISBR	Immobilized soil bioreactor
k_La	Mass transfer coefficient, h^{-1}
M	Solvent molecular weight
M_{CH4}	Molecular weight of methane
MMO	Methane monooxygenase
N	Total number of orifices

P_{CH4}	Partial pressure of methane in the headspace of reactor, atm
P/V	Volumetric power input, hp gallon^{-1}
PCE	Tetrachloroethene
PCP	Pentachlorophenol
pMMO	Particulate methane monooxygenase
RNApol	RNA polymerase
RuMP	Ribulose monophosphate pathway
S_t	Total amount of methanol to be fed at time t, g
SBR	Sequencing biofilm reactor
sMMO	Soluble methane monooxygenase
t	Time of fermentation, h
T	Absolute temperature, K
TCE	Trichloroethylene
UAS	Upstream activator
UV	Ultraviolet
V	Reactor volume, m^3
V_G	Headspace volume of each serum bottle, ml
V_L	Liquid phase volume of each serum bottle, ml
V_m	Molecular volume of solute at boiling point, cm^3 g^{-1} mol^{-1}
VC	Vinyl chloride
VOC	Volatile organic compound

X_o	Biomass when exponential feeding begins, g
X_t	Desired biomass at time t , g
$Y_{CO2/S}$	Yield of carbon dioxide from substrate, g CO2 g substrate-1
$Y_{X/S}$	Expected yield of biomass from substrate, g biomass g substrate-1

Greek letters

a	Specific surface area, m^{-1}
x_a	Constant, 2.6 for aqueous systems.
γ_{CH4}	Methane uptake rate, mg l^{-1} h^{-1}
μ	Desired growth rate, h^{-1}
μ_{CPR}	Specific growth rate based on carbon dioxide production, h^{-1}
μ_w	Dynamic viscosity of the liquid, Pa.s
ρ_w	Density of the liquid, kg m^{-3}

Chapter 1

Introduction

1.1 Background

Trichloroethylene (TCE), a volatile, chlorinated, organic compound, has been widely used as a solvent for degreasing in dry cleaning, electroplating, printing, pulp and paper, and the electronic industries for many years (ATSDR, 1997; WHO, 2004). TCE was once used as an inhalation anaesthetic during certain short surgical procedures (ATSDR, 1997; Slunge and Sterner, 2001). It has become a major contaminant in soil and groundwater due to its widespread use, inadequate disposal techniques, and persistence in the natural environment. It is among the most frequently detected contaminants in groundwater in Germany and the U.S.A. (Figure 1-1).

The release of TCE to the environment poses serious environmental problems through contamination of groundwater and soil. TCE is suspected of being carcinogenic and mutagenic and has been rated as a priority hazardous pollutant by the U.S.A. Environmental Protection Agency (EPA) (ATSDR, 1997). To protect our health, TCE is more and more strictly controlled in most countries. Organizations have set maximal contaminant levels for TCE in drinking water ranging from 0.005 to 0.07 mg l^{-1} (Table 1-1).

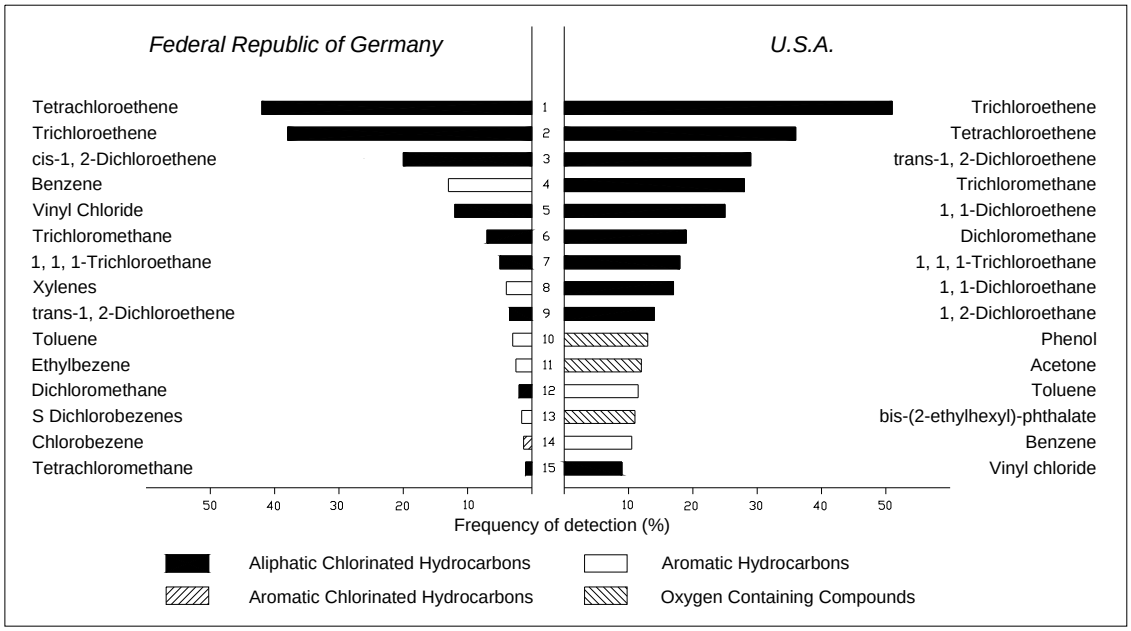

Figure 1-1 The 15 most frequently detected organic compounds in groundwater at waste disposal sites in Germany and the U.S. A. (reprinted from EPA/540/S-99/001).

At present, treatment processes for water contaminated with volatile organics include advanced oxidation and air stripping followed by activated carbon adsorption. However, air stripping-activated carbon adsorption transfers the organic contaminants to another phase without destroying them. Although, advanced oxidation can be used for complete elimination of the contaminant, it is not cost-effective. Due to increasing environmental concerns and federal regulations there is a growing demand for low-cost, highly effective treatment techniques for chlorinated solvents like TCE.

Table 1-1 Allowable TCE content in drinking water

Organization	Regulations and Guidelines	Allowable limit ($mg\ l^{-1}$)	Year
World Health Organization	Guidelines for drinking-water quality, 3rd edition	0.07	2004
Health Canada	Guidelines for Canadian Drinking Water Quality	0.005	2006
Ontario Ministry of Environment and Energy	Water Management Polices Guidelines Provincial Water Quality Objectives	0.02	1994
U.S. EPA	National Primary Drinking Water Regulations	0.005	2003

Microbial TCE degradation is receiving more interest as they present a possible cost-effective and efficient alternative to traditional treatment techniques. Bacterial TCE degradation may proceed as anaerobic dechlorination or aerobic cometabolism. During aerobic TCE cometabolism, TCE is degraded by bacteria expressing monooxygenases or dioxygenases. Substrates able to provide carbon and energy sources are always required during TCE cometabolism since the bacteria do not benefit from TCE degradation. A diverse range of bacteria has been reported to cometabolize TCE including *Pseudomonas*, *Nitrosomonas*, *Xanthobacter*, *Rhodococcus*, and methanotrophs (Fogel et al., 1986; Oldenhuis et al., 1989) (Table 1-2).

Table 1-2 Bacteria and enzyme activity involved in TCE cometabolism

Organisms	Enzyme	Degradation rate nmol min^{-1} mg cell $protein^{-1}$	Reference
Methylosinus trichosporium OB3b	sMMO	16.6	Koh et al., 1993
Methylomonas methanica 68-1	sMMO	38.75	Koh et al., 1993
Methylosinus trichosporium OB3b	sMMO	290	Oldenhuis et al., 1991
Pseudomonas cepacia G4	Toluene ortho-monooxygenase	8	Folsom et al., 1990
Methylocystis parvus OBBP	pMMO	0.677	Dispirito et al., 1992
Methylomonas sp. A45	pMMO	0.296	Dispirito et al., 1992
Methylomonas sp. MN	pMMO	0.233	Dispirito et al., 1992
Methylosinus trichosporium OB3b	pMMO	0.058	Dispirito et al., 1992
Methylosinus trichosporium OB3b	pMMO	2.5-4.1	Lontoh and Semrau, 1998
Methylococcus capsulatus (Bath)	pMMO	0.202	Dispirito et al., 1992
P. putida	Toluene dioxygenase	1.8	Wackett and Gibson, 1988
Nitrosomonas europaea	Ammonia monooxygenase	0.42	Arciero et al.,1989
Mycobacterium sp.	Propane monoxygenase	0.7*	Wackett et al., 1989
Alcaligenes eutrophus JMP 134	Phenol hydroxylase	0.2	Harker and Kim, 1990
Rhodococcus erythropolis	Isoprene oxygenase	0.2	Ewers et al., 1990

*Enzyme activity was measured as nmol min^{-1} mg $biomass^{-1}$

Among these bacteria, treatment systems based on the cometabolic transformation of TCE by methanotrophic bacteria are very promising due to the use of an inexpensive and non-toxic primary growth substrate, potentially high transformation rates, and applicability to a broad range of contaminants. Methanotrophs are characterized by their

utilization of a non-specific enzyme, methane monooxygenase (MMO), to catalyze the oxidation of methane to methanol. The soluble MMO (sMMO), produced mainly by type II methanotrophs, can cometabolize less-halogenated aliphatic compounds and aromatic compounds (Colby et al., 1977; Fogel et al., 1986; Fox et al., 1990). However, sMMO is only expressed by some methanotrophs under low copper conditions and trace amounts of copper is able to completely repress sMMO expression, while the other form of MMO, particulate MMO (pMMO), has little activity towards those compounds. Considering the fact that the presence of Cu in ground water and soil is inevitable, how to overcome the inhibitory effect of Cu on sMMO needs to be addressed. The other critical disadvantage of a methanotrophic system is the slow growth rate. These two limitations of a methanotrophic system actually impede the practical application and commercialization of methanotrophic TCE degradation over the last decade.

To study the performance of methanotrophs towards TCE degradation, several types of methanotrophic bioreactors have been developed including suspended growth reactors, biofilm reactors, and membrane reactors. A less well-studied reactor, the immobilized soil bioreactor (ISBR) provides an option for TCE biodegradation. The ISBR contains plates filled with polyester textile to facilitate soil entrapment and biofilm formation. Soil and its associated microorganisms are entrapped in the porous polyester textile. The primary substrate, methane, and nutrients are supplied to the ISBR to support methanotrophic growth and biofilm development in the reactor. The mixed methanotrophic culture also assimilates the toxic intermediates of TCE degradation and protects the primary TCE degraders, the methanotrophs. The ISBR allows for the

immobilization and development of methanotrophs at a lower cost than competitors such as membrane bioreactors.

1.2 Overall objective

The objective of this research is to develop an innovative, efficient and practical sMMO-based process, for TCE biodegradation in an ISBR.

1.3 Strategy

This research consisted of four phases as shown in Figure 1-2 to achieve the overall objective.

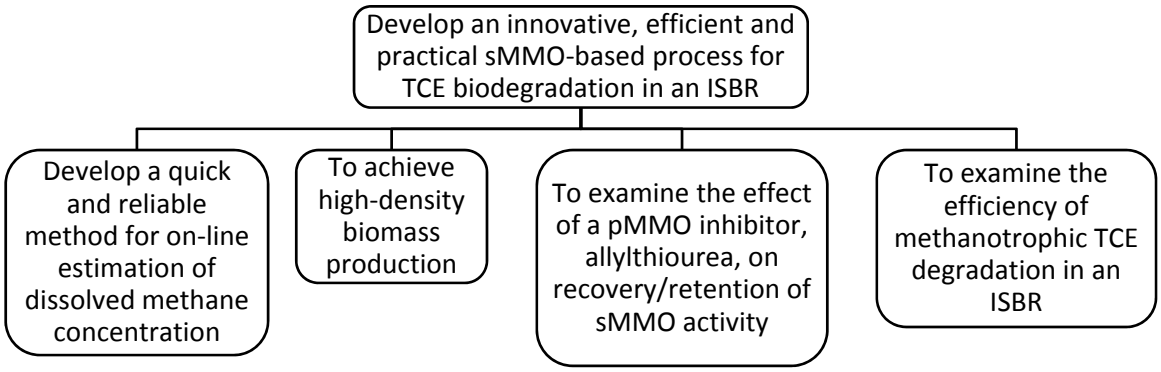

Figure 1-2 Flow chart of research objective and specific objectives

Phase I involved developing a method for on-line estimation of dissolved methane concentration which is critical to understand the kinetics of methanotrophic growth and cometabolic TCE degradation since methane serves as the growth substrate and competes with TCE for the active site on sMMO during cometabolism. Although a variety of methanotrophic bioreactors have been developed for TCE biodegradation, analysis of the aqueous phase methane was either ignored (Parvatiyar et al., 1996; Speitel and Leonard, 1992; Strandberg et al., 1989) or relied on estimating the dissolved

methane in the gas phase based on gas-liquid equilibrium (Arvin, 1991; Clapp et al., 1999; Fennell et al., 1993; Fitch et al., 1996; Lanzarone and Mccarty, 1990; Tschantz et al., 1995) which is time consuming and inconvenient. A method for continuous on-line estimation of dissolved methane was developed based on a mass balance and the mass transfer capacity of the reactor. This phase of study is covered in Chapter 3, "On-line estimation of dissolved methane concentration during methanotrophic fermentations", published in *Biotechnology and Bioengineering*, 95(5):788-793, 2006.

Phase II focused on high-density biomass production from methanol. In Phase I (Chapter 3), the data clearly showed that the low solubility of methane in the aqueous phase resulted in a poor mass transfer rate of methane in the bioreactor and consequently, poor methanotrophic growth. In order to circumvent this problem and achieve high cell density of a methanotroph (a pure culture of a typical type II methanotroph, *Methylosinus trichosporium* OB3b, was used as a model organism), a water miscible substrate, methanol, was evaluated. However, methanol is toxic to most bacteria. To avoid the toxic effects of methanol, feeding strategies based on fed-batch cultivation were used. This part of the research is covered in Chapter 4, "Production of soluble methane monooxygenase during growth of *Methylosinus trichosporium* on methanol", accepted by *Journal of Biotechnology*.

However high-density biomass production contributes to only one aspect of the successful application of methanotrophs to TCE degradation. High sMMO activity is also necessary. It is well recognized that Cu regulates the expression of sMMO and pMMO in Type II methanotrophs (Burrows et al., 1984; DiSpirito et al., 1992). Although different levels of Cu which completely or partially block sMMO activity have been

reported, all these values are below 1μM (0.064 mg l^{-1}) (Grosse et al., 1999; Jahng and Wood, 1996; Park et al., 1991; Tsien et al., 1989). Since Cu is normally present in the TCE-contaminated water, the negative effect of Cu on sMMO has to be overcome. In phase III, the use of a pMMO inhibitor, allylthiourea, was applied to overcome the negative effect of Cu on sMMO activity. The effect of allylthiourea on sMMO expression in the presence of Cu was investigated with a pure culture of *Methylosinus trichosporium* OB3b. This phase of research is covered in Chapter 5, "Use of allylthiourea to produce soluble methane monooxygenase in the presence of copper", submitted to Applied Microbiology and Biotechnology.

The achievements of phase II, Chapter 4, and phase III, Chapter 5, were applied to the treatment of TCE-contaminated water in an ISBR. Methane and allylthiourea were used for the selective enrichment of sMMO-producing methanotrophs. Then methanol and the pMMO-inhibitor were fed to obtain high-density biomass with sMMO activity. TCE degradation was performed successfully in batch and continuous mode. ISBR operation was alternated between methanotrophic growth/sMMO regeneration and TCE degradation. Once the sMMO activity had deteriorated, methanol or methane feed was resumed for biomass regeneration and sMMO recovery. This phase of the research is covered in Chapter 6, "Biodegradation of trichloroethylene in an immobilized soil bioreactor", to be submitted to *Biodegradation*.

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Chapter 2

Literature Review

2.1 Groundwater treatment technologies

Several technologies are presently available for the removal of volatile organic compounds (VOCs) from groundwater. These techniques usually involve pump-and-treat processes, where the groundwater is pumped to the surface, treated and returned to the aquifer or discharged. Air stripping followed by carbon adsorption, and advanced oxidation are among the most common treatments used in such processes.

2.1.1 Air stripping and active carbon adsorption

Air stripping has been widely accepted as a process to remove VOCs from groundwater or surface water. One type of air stripper is a packed tower which usually consists of a large tank filled with packing material (U.S. EPA, 2001b). The contaminated water is introduced from the top. While the water is sprayed over the packing material and trickles down, air is forced through the trickling water to evaporate the contaminants. The ascending air carries the contaminants and exits at the top of the tank. The off gas containing the volatilized compounds may require further treatment before being discharged to the atmosphere. Clean water is collected at the bottom. Other types of air strippers include aeration tanks and tray strippers (Bazzazieh, 1996). All air strippers aim to increase air-water contact and facilitate the mass transfer of VOCs from aqueous phase to air. The Henry's law constant determines the efficiency of air stripping for organic compounds.

Activated carbon adsorption is a widely used technology for removing organic contaminants from groundwater. Active carbon adsorption functions as a filtration system. A liquid or gas stream is pumped through a series of vessels containing activated carbon (Bazzazieh, 1996; U.S. EPA, 2001a). The porous structure of activated carbon provides a large specific surface area and allows the adsorption of relatively large quantities of contaminants. The method is especially effective for compounds with low polarity. Carbon adsorption can be used as a stand-alone treatment or in conjunction with other processes for polishing. Activated carbon must be replaced or regenerated when the adsorptive capacity has been saturated and contaminants breakthrough occurs.

Although air stripping and carbon adsorption are widely applied to groundwater decontamination of organic compounds, these are basically physical processes and simply transfer contaminants from one phase to another without eliminating the contaminants.

2.1.2 Advanced oxidation

In the past years, there has been considerable research in the application of advanced oxidation process (AOP) for the treatment of organic contaminants in water and vapor phases (Guha et al., 1995; Karimi et al., 1997; Sunder and Hempel, 1997; Weir et al., 1996). AOPs use a combination of ultraviolet (UV), ozone, and hydrogen peroxide to completely oxidize organic pollutants to carbon dioxide and water. These processes have shown considerable potential in the treatment of recalcitrant organic pollutants as diverse as humic substances, textile dye waste, and sewage sludge. The main advantage of AOP is that it destroys the contaminants instead of transferring them to another phase

as with air stripping or carbon adsorption. However, AOPs are energy intensive and require the addition of expensive chemicals.

2.1.3 Biological treatment

The cleanup technologies discussed in the preceding sections are not satisfactory in treating TCE-contaminated groundwater. Hence it is necessary to develop novel technologies to remediate contaminated groundwater more effectively, less expensively, and in a manner more acceptable to the public than existing conventional methods. Bioremediation has been receiving more interest in recent years. This approach has been demonstrated with *in situ* and *ex situ* treatment of TCE-contaminated groundwater (Roberts et al., 1990; Semprini et al., 1990; Sutfin and Ramey, 1997; Travis and Rosenberg, 1997).

Biological degradation of chlorinated aliphatic hydrocarbons (CAHs) like TCE can proceed by two general mechanisms: aerobic cometabolism and anaerobic dechlorination.

2.1.3.1 Anaerobic biodegradation

One approach to CAH biodegradation is an anaerobic process which is known as reductive dechlorination (Bouwer et al., 1981) where chlorinated compounds are used as electron acceptors and dechlorinated stepwise. For example, TCE is dechlorinated to form dichloroethenes (DCEs) then vinyl chloride (VC) and finally ethylene (Figure 2-1). A serious concern of this process is that TCE may only be partially dechlorinated to VC which is more toxic and carcinogenic (Carter and Jewell, 1993; Freedman and Gossett, 1989; Vogel and McCarty, 1985) than the parent compound. Therefore, anaerobic

treatment as a single system is not practical unless the process results in complete dechlorination to ethylene.

2.1.3.2 Cometabolism of TCE

Some of the less chlorinated solvents, such as DCE (Vandenwijngaard et al., 1992), and VC (Hartmans and Debont, 1992; Verce et al., 2002) may also serve as sole sources of carbon; however, the heavily chlorinated compounds, such as tetrachloroethene (PCE) and TCE, are quite resistant to biodegradation, since they cannot serve as an energy source and hence do not support microbial growth. Aerobic TCE degradation may occur through cometabolism, in which microorganisms degrade these compounds without gaining any benefit while growth is supported by some other readily metabolizable substrate. Furthermore, formation of undesirable partially dechlorinated byproducts is avoided in aerobic TCE degradations, such as cis-DCE, trans-DCE or VC.

TCE can be degraded by a diversity of bacteria including methanotrophs, and species of *Pseudomonas* (*P. putida* and *P. cepacia*) (Cox et al., 1998; Folsom et al., 1990; Landa et al., 1994; Shields and Reagin, 1992). The common feature of these microorganisms is the use of a primary substrate such as methane (Alvarez-Cohen et al., 1992; Broholm et al., 1993; Fogel et al., 1986; Mcfarland et al., 1992; Tsien et al., 1989; Wilson and Wilson, 1985), phenol (Bielefeldt and Stensel, 1999; Folsom et al., 1990; Kim, 1997), toluene (Cox et al., 1998; Landa et al., 1994; Leahy et al., 1996; Parvatiyar et al., 1996; Shields and Reagin, 1992), propane (Wackett et al., 1989), or ammonia (Vannelli et al., 1990) to induce a non-specific oxygenase that can also catalyzes the degradation of CAHs. The pitfall for a cometabolic process is that the non-growth substrate must compete with a growth substrate for a specific active site of the oxygenase enzyme.

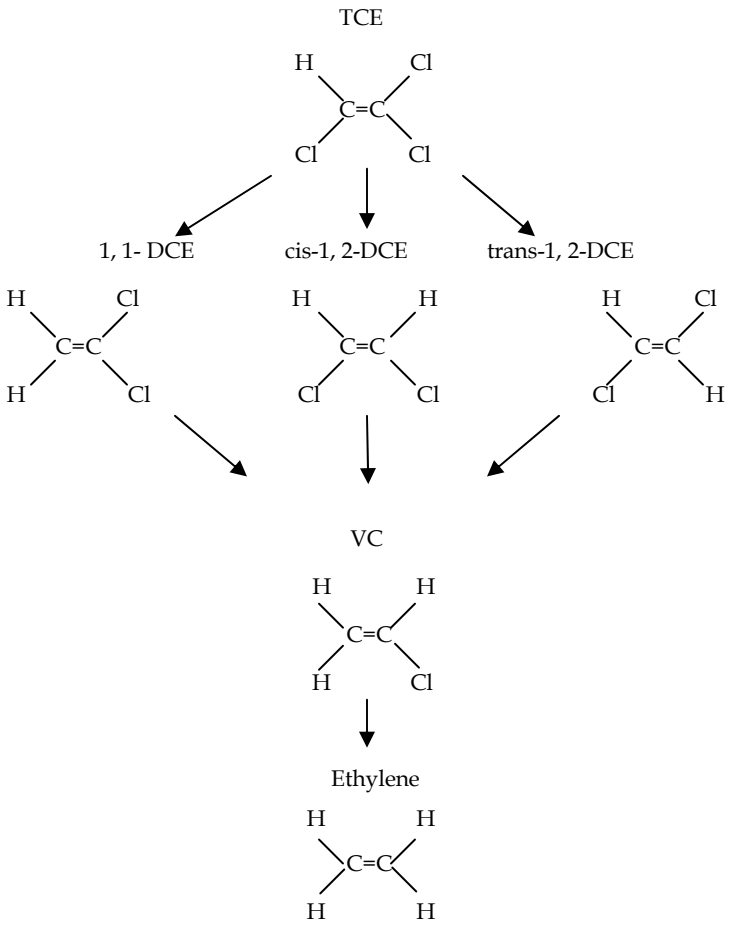

Figure 2-1 Anaerobic degradation pathways for trichloroethylene (adapted from EPA/540/S-99/001).

Although microorganisms using ammonia, toluene, and phenol as growth substrates show promising results in cometabolizing TCE, these substrates are of serious environmental concerns. Ammonia is an environmental pollutant, while aromatic compounds are toxic and regulated chemicals, limiting their application in the environment. In the presence of oxygen and methane, methanotrophic bacteria produce MMO (Hanson and Hanson, 1996). The primary substrate for this enzyme is methane, but it has been shown to have broad substrate specificity (Burrows et al., 1984).

Methanotrophs are of interest because they use a nontoxic and widely available substrate, and have higher specific TCE oxidation rates than other TCE degraders. The ability of methanotrophs to use methane as a growth substrate and simultaneously cometabolize CAHs through MMO has allowed for a relatively efficient and inexpensive approach for bioremediation of TCE.

2.2 Methanotrophic degradation of TCE by cometabolism

2.2.1 Introduction to methanotrophs

Methanotrophic bacteria are ubiquitous in the environment. They have been detected in swamps, rivers, oceans, and sewage sludge (Hanson and Hanson, 1996). Methanotrophic genera include *Methylococcus*, *Methylomonas*, *Methylomicrobium*, *Methylobacter*, *Methylocystis* and *Methylosinus*. Three physiological groups of methanotrophs can be distinguished mainly based on metabolic pathways, physiochemical properties, phospholipid fatty acid composition, and membrane structure.

Type I methanotrophs (*Methylomonas*, *Methylomicrobium*, *Methylobacter*) are distinctive in using the ribulose monophosphate pathway (RuMP) to assimilate the formaldehyde produced from methane oxidation, having cellular membranes composed of predominantly 16-carbon fatty acids and possessing bundles of intracytoplasmic membranes (Hanson and Hanson, 1996). Type II methanotrophs (*Methylocystis* and *Methylosinus*) utilize the serine pathway for formaldehyde assimilation, have intracytoplasmic membranes arranged around the periphery of the cell and contain a high level of 18-carbon fatty acids. The genus *Methylococcus* possesses a combination of

characteristics of both Type I and Type II methanotrophs and are classified as Type X. Analysis of 16S RNA shows that Type I and Type X methanotrophs belong to γ-subdivision of the *Proteobacteria* and Type II methanotrophs are grouped within the α-subdivision of *Proteobacteria* (Bowman et al., 1993; Hanson and Hanson, 1996). The differences of three types of methanotrophs are summarized in Table 2-1.

Table 2-1 Comparisons of type I, type II, and type X methanotrophs (Hanson and Hanson, 1996)

	Type I	Type II	Type X
Growth at 45℃	No	No	Yes
G+C content of DNA, %	49-60	62-67	59-65
Intracellular membrane			
Stacked	Yes	No	Yes
Peripheral	No	Yes	No
Nitrogenase activity	No	Yes	Yes
Metabolic pathway			
RuMP	Yes	No	Yes
Serine	No	Yes	Sometimes
Major phosphor lipid fatty acids	14:0, 16:1ω7c, 16:1ω5t	18:1ω8c	16:0, 16:1ω7c,
Proteobacterial subdivision	γ	α	γ

Wilson and Wilson (1985) first demonstrated methanotrophic biodegradation of TCE in sand column studies where indigenous methanotrophic microorganisms were biostimulated by feeding natural gas. TCE degradation to carbon dioxide coincided with an increase in methanotrophic population. Later, Fogel et al. (1986) further indicated the potential of TCE biodegradation based on methanotrophic cometabolism by methane-

utilizing organisms grown in suspension culture. Since then significant progress has been made in the application of methanotrophic bacteria to bioremediation (Broholm et al., 1990; Little et al., 1988; Oldenhuis et al., 1989; Strandberg et al., 1989; Tsien et al., 1989).

2.2.2 Mechanism of TCE degradation by methane monooxygenase

MMO catalyzes the conversion of methane to methanol, the first step in the pathway for methane utilization (Figure 2-2). MMO is also capable of inserting oxygen into TCE at the carbon-carbon double bond and creating an unstable epoxide (Figure 2-2). The TCE epoxide breaks down to formate, dichloroacetic acids, and glyoxylate. Methanotrophs and other heterotrophs will assimilate these intermediates to produce biomass and release carbon dioxide.

Complete transformation of TCE to carbon dioxide relies on the activity of other kinds of heterotrophs. Uchiyama et al. (1992) isolated these heterotrophs and identified them as *Xanthobacter* and *Pseudomonas*. Application of a mixed culture in TCE degradation could lead to a higher TCE degradation capacity by reducing the accumulation of toxic intermediate products.

It has been suggested that TCE degradation is catalyzed not only by epoxidation but also by a Cl shift followed by the formation of trichloroacetic acid and 2,2,2-trichloroethanol, which is supported by the presence of trichloracetic acid in TCE degradation intermediates (Uchiyama et al., 1992).

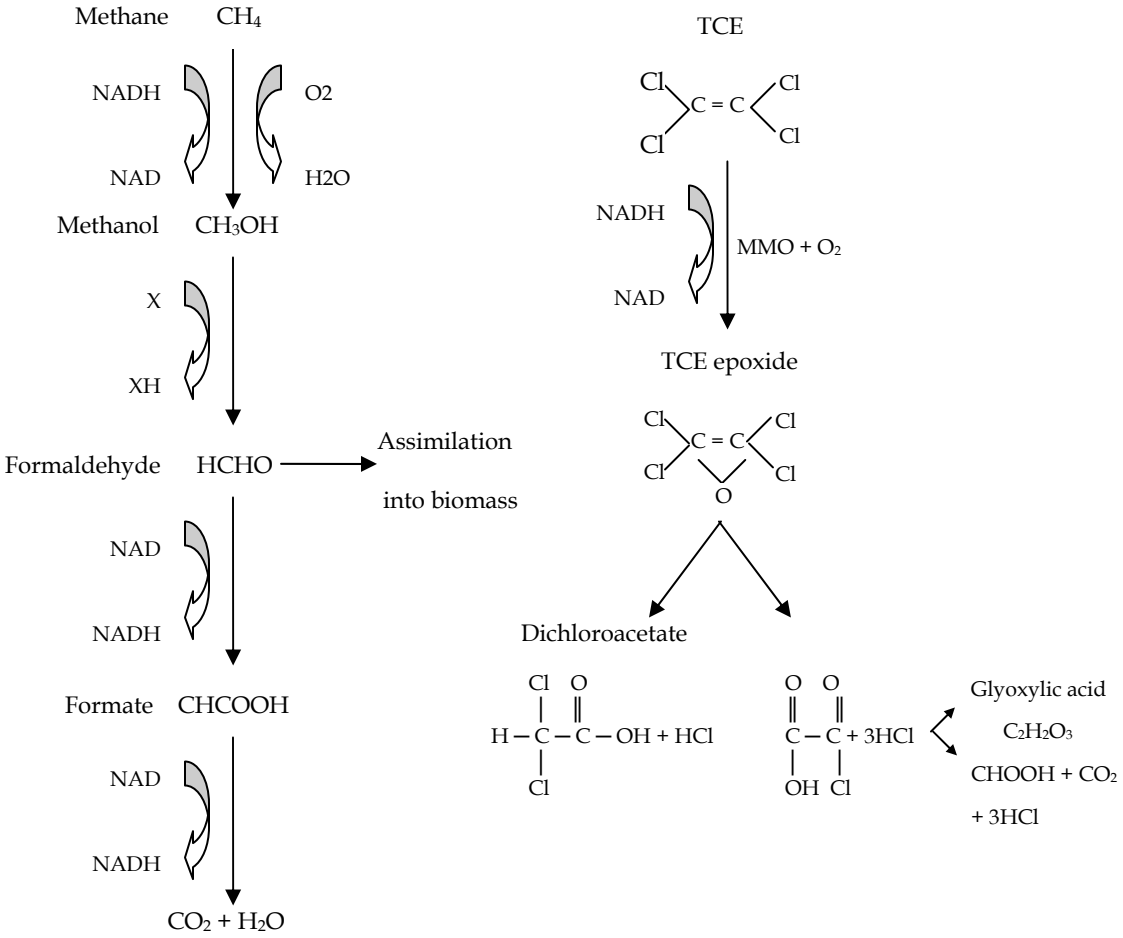

Figure 2-2 Oxidation of methane and proposed mechanism for TCE cometabolism by MMO (U.S. DOE, 2001).

2.2.3 Classification of methane monooxygenase

MMO exists in two forms: the cytoplasmic, soluble from (sMMO) and the membrane-bound, particulate form (pMMO) (Burrows et al., 1984; Scott et al., 1981; Stanley et al., 1983). All methanotrophs express pMMO, while sMMO is expressed mainly by Type II and Type X methanotrophs under copper deficient conditions (Bowman et al., 1993; Grosse et al., 1999; Stanley et al., 1983). Two acidophilic methanotrophs, *Methylocella palustris* and *Methylocella silvestris*, were isolated recently and found to express sMMO

constitutively (Dedysh et al., 2000; Dunfield et al., 2003). Other exceptions include two type I methanotrophs, strain 46-1, and *Methylomonas methanica* 68-1 which were reported to be able to degrade TCE (Little et al., 1988; Shen et al., 1997).

sMMO and pMMO are not only different in structure and catalytic ability, but also in substrate specificity. sMMO can degrade a wider variety of compounds, such as chlorinated aliphatic compounds and aromatics (Colby et al., 1977; Hesselsoe et al., 2005). Although pMMO has been reported to degrade CAHs, only sMMO-expressing methanotrophs exhibit practical TCE degradation rates. TCE degradation rates with sMMO-expressing methanotrophs was determined to be 27~150 nmol min^{-1} per mg of cell from *M. trichosporium* OB3b at 30℃ and 3.2~11.1 nmol min^{-1} per mg of cell from a mixed culture at 21℃ (Alvarez-Cohen and McCarty, 1991; Oldenhuis et al., 1989), which are two or more orders of magnitude higher than the rates reported with pMMO which varied from 0.001 - 0.68 nmol min-1 per mg of cell protein measured at room temperature (DiSpirito et al., 1992).

sMMO has been purified from *M. trichosporium* OB3b, and *Methylococcus capsulatus* Bath (Fox et al., 1989). The sMMO from these microorganisms is highly conserved in its structure. sMMO contains three components: hydroxylase (245kDa), B component (15.8kDa), and reductase (38.4kDa). The reductase contains a FAD and a [2Fe-2S] cluster and functions as a moderator for hydride transfer from NADH to hydroylase. The component B has no cofactors or metals and may serve as an electron transfer effector. The 245-kDa hydroxylase contains an oxo- or hydroxyl-bridged binuclear iron cluster which appears to be the active site of monooxygenase reaction. Mg, Cu, Co, Mo and Ni were not found in the hydroyxylase (Fox et al., 1989). The sMMO from *Methylocystis sp*.

WI14 was also purified (Grosse et al., 1999) and found to consist of three polypeptide of 229, 41, and 18 kDa. The hydroxylase has three subunits of 57, 43, and 23 kDa arranged in $(\alpha\beta\gamma)_2$ structure. It was found that each mole of hydroxylase contains 3.6 mol of iron in its active sites. (Grosse et al., 1999). The structure of the sMMO molecule has been described (Murrell et al., 2000a).

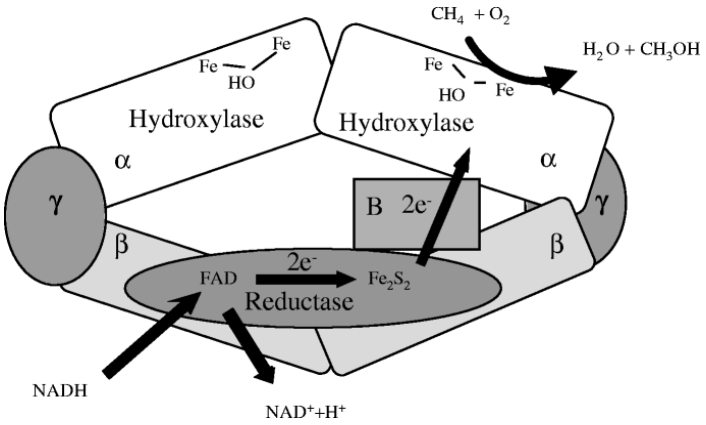

Figure 2-3 Model of the sMMO complex proposed by Murrell et al. (2000a).

By comparison, three polypeptides with molecular weights of 43~47, 34~35, and 26 kDa were isolated from the membrane fractions of methanotrophs expressing pMMO (DiSpirito et al., 1992; Stanley et al., 1983; Zahn and DiSpirito, 1996). pMMO is believed to have copper present in the active site (DiSpirito et al., 1992; Nguyen et al., 1994; Semrau et al., 1995). Zahn and DiSpirito (1996) quantitatively determined that pMMO contained 2.5 iron atoms and 14.5 copper atoms per 99 kDa. The differences in molecular structure demonstrate that the conversion from sMMO to pMMO is not only a result of changing the location of the enzyme in the cells, but may also involve a complicated biochemical process.

pMMO and sMMO are also different in their inhibition characteristics. pMMO is sensitive to a variety of inhibitors including metal chelators, thiol reagents, KCN, and pyridine compounds (Bedard and Knowles, 1989; Hubley et al., 1975; Scott et al., 1981; Stanley et al., 1983). The inhibitory effect applies to pMMO from both type I and type II methanotrophs. Among these inhibitors, supplementation of several metal ions such as Fe^{2+}, Cu^{2+}, Co^{2+}, and Zn^{2+} is able to reduce the inhibition of some compounds, but the inhibition by thiourea and allylthiourea is not relieved by any of the metals (Hubley et al., 1975).

2.2.4 Effect of copper on regulation of sMMO

It is well recognized that copper regulates the expression of sMMO and pMMO in Type II methanotrophs (Dalton et al., 1984; Nielsen et al., 1996; Nielsen et al., 1997; Stanley et al., 1983). In copper-sufficient media, pMMO is preferentially expressed (Burrows et al., 1984; Stanley et al., 1983). Concentrations of copper as low as 1 μM have been reported to completely inhibit sMMO expression (Koh et al., 1993; Park et al., 1992). This is consistent with Grosse et al. (1999) who found that 0.1 μM of copper would result in 60% loss of sMMO activity while Jahng and Wood (1996) reported 0.4 μM copper fully repressed sMMO activity in *M. trichosporium* OB3b. Tsien et al. (1989) also found that 0.25 μM of copper (0.016 mg l^{-1}) depressed the synthesis of sMMO and reduced TCE degradation rates. The addition of copper also resulted in the formation of intracytoplasmic membranes (Phelps et al., 1992).

Copper may affect sMMO activity at the genetic level. Copper was found to regulate MMO expression during gene transcription (Murrell et al., 2000a; Murrell et al., 2000b). Upon availability of copper, sMMO-specific mRNAs in *Methylococcus capsulatus*

Bath and *M. trichosporium* OB3b decreased and disappeared in 10-30 min after copper addition. The induction of *pmo* gene cluster transcription to pMMO specific mRNA was also activated. It was assumed that copper combined with a transcriptional regulator and regulated gene expression by binding to the *smmo* or *pmo* operator. The model of copper-dependent transcription regulation of *pmo*/*smmo* genes proposed is described in Figure 2-4 (Nielsen et al., 1996; Nielsen et al., 1997). The regulator R represses *pmo* gene transcription by binding to the *pmo* operator under copper-free conditions. The regulator also binds to the upstream activator (UAS) of the *smmo* promoter and RNA polymerase (RNApol) to activate *smmo* gene transcription. Excess copper will change the conformation of the regulator so it will no longer be able to bind to the *smmo* or *pmo* promoters. Therefore the *pmo* gene is derepressed and the *smmo* gene is not transcribed.

Copper not only depresses sMMO expression at the genetic level, but also inhibites the sMMO activity. Like other heavy metals, copper can be a potent enzyme inhibitor. As to sMMO, sMMO inactivation is achieved by interaction of copper with the reductase component *in vitro* (Green et al., 1985). When the copper concentration is high, copper interacts with reductase and disrupts the protein structure, causing the interruption of the Fe-S active sites and block of electron transfer from the reductase to the hydroxylase, while the hydroxylase and component B are not affected. The inactivation of reductase is irreversible. Jahng and Wood (1996) found that inactivation of sMMO by copper was the result of inhibition of both the hydroxylase and reductase.

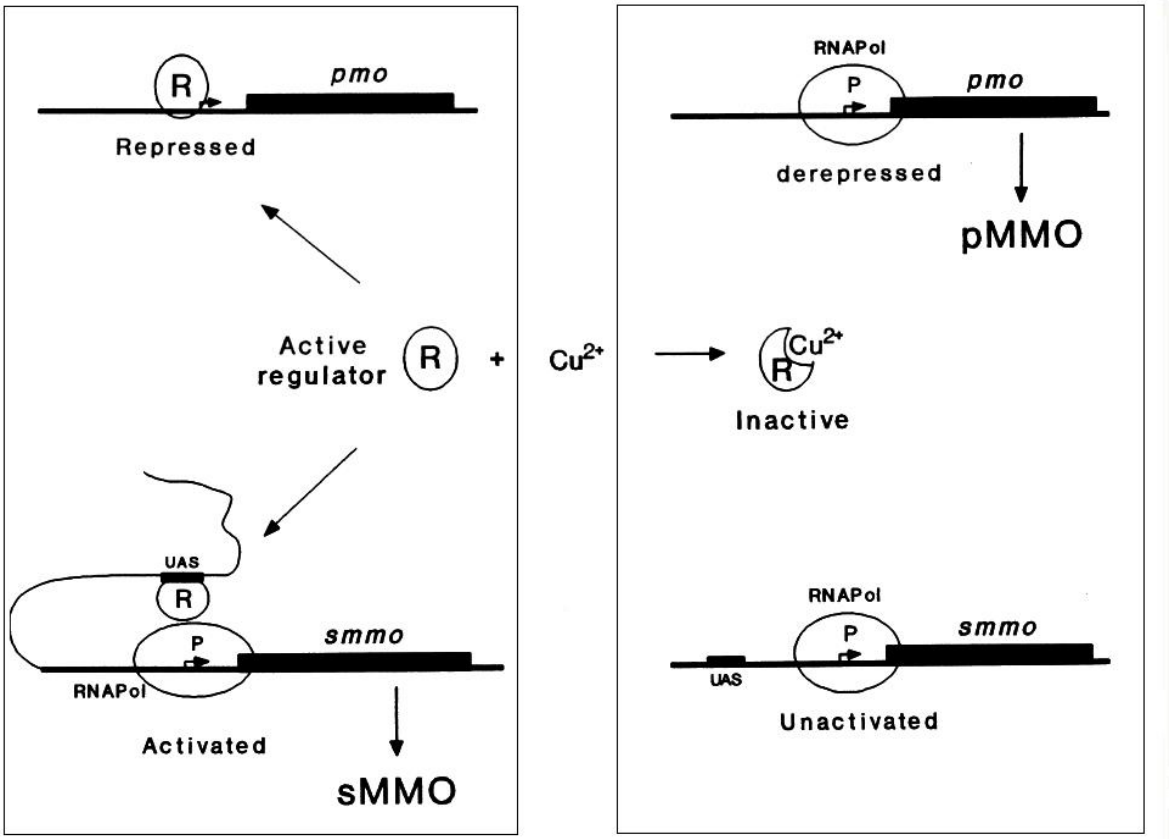

Figure 2-4 Regulation of *pmo*/*smmo* genes expression by copper (Nielsen et al., 1997).

The fact that a copper content as low as 0.1 μM may have a significant negative effect on sMMO activity strongly limits application of methanotrophs to TCE bioremediation since the presence of copper in nature is inevitable. A survey of Canadian surface and lake waters from 1980 to 1983 indicated that extractable copper levels ranged from 0.001 to 0.080 mg l^{-1} (0.016~1.259 μM), although concentrations rarely exceeded 0.005 mg l^{-1} (0.079 μM) (CCME, 1987). Méranger et al. (1979) also measured the copper concentration in drinking water supplies (Table 2-2).

Table 2-2 Copper concentrations in Canadian drinking water supplies (Meranger et al., 1979)

Water source	Raw water μg l^{-1}		Treated water μg l^{-1}		Distributed water μg l^{-1}	
	Mean	Range	Mean	Range	Mean	Range
River	≤5	≤5-530	≤5	≤5-100	20	≤5-220
Lake	≤5	≤80	≤5	≤5-100	40	≤5-560
Well	≤5	≤110	≤5	≤5-70	75	10-260

In areas where waste from mining and other copper industries are disposed, high concentrations are inevitable (ATSDR, 1990). It is possible that the copper concentration in groundwater will be high enough to inhibit the expression of sMMO. Even low concentrations could cause significant problems in a methanotrophic reactor.

The requirement for the absence of copper to induce the production of sMMO and high TCE degradation rates can be a handicap for bioremediation. To overcome this problem, mutant strains of *M. trichosporium* OB3b, which lack the ability to express pMMO but are able to express sMMO constitutively even in the presence of up to 12 μM of copper have been developed (Fitch et al., 1996a; Phelps et al., 1992). Tschantz et al. (1995) successfully used one of these mutant strains (*M. trichosporium* OB3b PP358) to degrade 0.2-20 mg l^{-1} TCE in a two-stage bioreactor for up to two weeks. Studies on the copper-binding compounds (CBCs) indicate that the mutation may be in the copper acquisition system (DiSpirito et al., 1998). However, unlike wild-type strains, these mutant strains have particular nutritional requirements, such as yeast extract and vitamins to maintain cell activity. Antibiotics were also needed to prevent contamination.

2.3 Methanotrophic bioreactors

Many methanotrophic bioreactors used in a pump-and-treat process have been developed in recent years. These reactors can be divided into two groups, single-stage or multi-stage, in terms of the bioreactor configurations. Although the ISBR should be considered as a type of single-stage reactor, it is discussed in a separate section. Some of the bioreactor systems are compared according to their performance, operating conditions, and the microbial cultures (Table 2-5, Table 2-6). These values helped in the selection of operating conditions in the ISBR.

2.3.1 Single-stage biofilm bioreactors

Several systems have been proposed for methanotrophic cometabolic TCE degradation within one reactor. These include expanded-bed reactors (Fennell et al., 1993; Phelps et al., 1990; Phelps et al., 1991), packed-bed biofilm reactors (Fitch et al., 1996b; Speitel and Leonard, 1992; Speitel and Mclay, 1993; Speitel and Segar, 1995; Strandberg et al., 1989), fluidized-bed reactors (Shimomura et al., 1997), membrane-attached biofilm reactors (Clapp et al., 1999), and a rotating cylinder biofilm reactor (Arvin, 1991). In all these bioreactors, methanotrophs are either entrapped in beads or attached to some support media to retain the cells within the reactors. Among these studies, three reactors were operated as sequencing biofilm reactors (Fitch et al., 1996b; Speitel and Leonard, 1992; Speitel and Segar, 1995).

2.3.1.1 Packed-bed and expanded-bed reactors

The support matrix used in methanotrophic bioreactors to facilitate bacterial attachment include activated carbon (Fennell et al., 1992; Fennell et al., 1993), diatomaceous earth

(Fennell et al., 1992; Fennell et al., 1993), glass (Phelps et al., 1990), and ceramic packing material (Speitel and Mclay, 1993; Strandberg et al., 1989). Usually before the treatment, the bioreactors are seeded with pure or mixed cultures of methanotrophs. Methane, air, and nutrients are supplied to allow the growth of biofilm onto the support matrix. TCE-containing water is passed through the reactor for biodegradation by microorganisms attached to the packing media.

TCE degradation was investigated in a 2.16-l methanotrophic packed column (Strandberg et al., 1989). The microbial consortium was obtained from a TCE-contaminated groundwater monitoring well. After 3-4 weeks, a salmon-pink colored biomass was visible throughout the column. However, the biofilm did not attach tightly to the column matrix. The reactor degraded 40 to 80% of influent TCE (1.0 mg l^{-1}) at residence times of 11-75 min.

A pure culture of *M. trichosporium* OB3b was grown in a 1.77-l packed-bed reactor to treat gas streams containing 327 μg l^{-1} TCE (Speitel and Mclay, 1993). TCE degradation averaged 62.3% at a gas residence time of 9.9 min. However, methanotrophic TCE degradation by a mixed culture in a small (65 ml) expanded-bed reactor (Niedzielski et al., 1989; Phelps et al., 1990) removed 50% TCE at influent TCE concentration of 20 mg l^{-1}.

Fennell designed a 3-l methanotrophic expanded-bed reactor and used primary sewage and soil from a natural gas saturated area as an inoculum (Fennell et al., 1992). Within 18 days of operation, a salmon-colored biofilm was observed on the granular activated carbon. Influent TCE concentration ranges from 0.56-13 mg l^{-1} in batch runs

and 0.1-40 mg l^{-1} in continuous runs. TCE removal efficiencies varied from 25 to 61% at hydraulic retention time of 1.2 to 11 h.

For both packed column and expanded-bed reactors, a mixture of methane and air was used. Large volumes of methane and air mixtures could pose a significant safety problem. Fennell et al. (1992) separated the feed of methane and air to reduce the explosion hazard. Nevertheless this problem has not yet been completely overcome.

The growth substrate, methane, can be a competitive inhibitor for TCE degradation in most operations since growth of methanotrophs and TCE degradation must occur simultaneously. sMMO must be induced by its growth substrate. These nonspecific enzymes bind to both the growth and to the cometabolic substrate. The half-saturation constants for methane and TCE by some methanotrophic cultures are summarized (Table 2-3 and Table 2-4). Methanotrophs have higher affinity to methane in terms of mg l^{-1}. Hence, even a low amount of methane may significantly inhibit TCE degradation and this competitive inhibition limits the TCE degradation rate in a single-stage reactor. It was observed that an increase in dissolved methane concentration from 0.01 mg l^{-1} to 5 mg l^{-1} reduced the TCE degradation rate from 2.5 mg g VS^{-1} d^{-1} to 0.3 mg g VS^{-1} d^{-1}. Optimal TCE degradation was maintained when the headspace methane content was kept below about 2% and a dissolved methane concentration of 0.5 mg l^{-1} (Fennell et al., 1993).

Table 2-3 Half-saturation constant for methane from some methanotrophic cultures

Microorganism	Ks , μM	Reference
Methylocystis strain LR1	2.2-12.6	Dunfield and Conrad, 2000
M. trichosporium OB3b	2	Joergensen, 1985
Methylococcus NCIB 11083	40	Linton and Vokes, 1978
M. trichosporium OB3b	92±30	Oldenhuis et al., 1991
M. trichosporium OB3b	18.75	Speitel et al., 1993

Table 2-4 Half-saturation constant for TCE from some methanotrophic cultures

Microorganism	Ks , μM	Reference
Mixed culture	55.5	Alvarez-Cohen and McCarty, 1991
Mixed culture	14.8±3.5	Chang and Criddle, 1997
Mixed culture	21.3	Fennell et al., 1993
M. trichosporium OB3b	45	Kang et al., 2001
M. trichosporium OB3b	145±61	Oldenhuis et al., 1991

To minimize competitive inhibition, some single-stage reactors limited methane concentration in the gas phase to be less than 5% (Arvin, 1991; Fitch et al., 1996b; Phelps et al., 1990; Speitel and Mclay, 1993; Strandberg et al., 1989).

To obtain high TCE removal efficiencies, improvements in reactor design and operation strategies are needed to maximize MMO production and minimize competitive inhibition. Examples of such strategies include alternately feeding either methane or TCE to a single reactor or using multiple-stage reactors where methanotrophic growth and TCE degradation are separated.

2.3.1.2 Membrane-attached biofilm reactor

A membrane-attached methanotrophic biofilm reactor was developed for the cometabolic degradation of TCE (Clapp et al., 1999). In this reactor, a mixed culture of methanotrophic biofilm was cultivated on the external surface of gas-permeable silicone tubing. Methane and oxygen were supplied to the lumen of the tubing, while contaminated water passed through the shell. The interior biofilm was protected from TCE and was in contact with methane and oxygen, to sustain the growth of the methanotrophs. Methane concentration was lower near the exterior of the biofilm which was exposed to TCE-contaminated water. This reactor facilitated a counter-diffusion to minimize competitive inhibition between methane and TCE. The biofilm inactivated by TCE or its byproducts can also be replaced by new cells. A high content of methane (25% v/v) was used to treat 4.6 ppm TCE with 80-90% TCE removal efficiency maintained over 18 weeks.

2.3.1.3 Sequencing biofilm reactors

Another approach to the minimization of competitive inhibition is a sequencing biofilm batch reactor. Sequencing biofilm reactors (SBRs) minimize competitive inhibition by cyclical operation of a single reactor in growth and degradation modes, in which the methane and TCE-containing stream are fed alternately.

Methanotrophic sequencing biofilm reactors were first applied to the bioremediation of TCE and related compounds by Speitel and coworkers (Speitel and Leonard, 1992; Speitel and Segar, 1995). They found that TCE degradation was enhanced when the influent was supplemented with formate. In this case, the degradation rate

remained stable for several days, while in the absence of formate, the initial degradation rate was lower and declined more rapidly. It is believed that NADH is regenerated during the oxidation of formate to carbon dioxide. This reaction does not involve the activity of sMMO, hence avoids enzyme competition.

Fitch et al. (1996b) designed a column packed with diatomaceous earth or glass beads on which a biofilm of mutants of *M. trichosporium* OB3b were developed in a growth cycle in which methane and air were fed for 3 to 38 days until biomass developed, as indicated by CO_2 evolution. The columns were subsequently operated in a degradation cycle, in which the column was completely filled with TCE-contaminated liquid. TCE degradation was sustained only for a short time (typically 24 to 48 h), and recycling back to growth mode did not completely regenerate the TCE degradation capacity.

A novel SBR was proposed by Shimomura et al. (1997). Methanotrophs (*Methylocystis sp*. strain M) were immobilized in 2% calcium alginate beads for TCE degradation in a fluidized bed reactor (FBR). The FBR was operated in a cycle of degradation and rejuvenation. During degradation, a relatively low concentration of methane (10% v/v) was fed to the reactor. Every 48 hours, the TCE feed was interrupted and a higher concentration of methane (20% v/v) was fed to rejuvenate the population. This 20 l FBR reduced the TCE concentration from 0.9-1.6 mg l^{-1} to 0.1-0.2 mg l^{-1} at a residence time of 2.56 h. However after 15 days of operation, the degradation efficiency declined and rejuvenation was increasingly ineffective.

2.3.2 Two-stage bioreactor

In the two-stage reactors, growth on the primary substrate (methane) and TCE degradation take place in separate reactors. Since the resting cells of methanotrophic bacteria have the ability to transform TCE (in the absence of methane), competitive inhibition between the growth substrate and contaminant is avoided.

Alvarez-Cohen and McCarty (1991) and Smith and McCarty (1997) have proposed, tested, and modeled a TCE two-stage treatment system. The methanotrophs were grown in suspension in a first-stage CSTR and the second stage was a plug flow reactor fed with TCE-contaminated groundwater and effluent from the growth reactor.

A hollow fiber membrane bioreactor has also been used in the second stage (Aziz et al., 1995; Pressman et al., 1999; Pressman et al., 2000). In methanotrophic TCE degradation, contaminated water or gas flowed through the lumen of the porous fibers and TCE diffused through the micropores in the membrane to the shell side where it was degraded by the flowing cell suspension. The advantage of this design is that biomass does not mix with the contaminated water. Hence, no separation step is necessary to remove the biomass.

Although two-stage systems overcome the disadvantage of competitive inhibition between methane and TCE, they present a new challenge. Similar to SBRs, the main problem is that TCE oxidation does not regenerate NADH. NADH is a coenzyme that has the ability to take up protons and high energy electrons from oxidation reactions. NADH then goes to the electron transport chain, where the two electrons are released and NADH returns as NAD^+. The high energy from the electrons is transferred to ATP. Therefore, reducing equivalents may become limiting in the second (conversion) reactor.

It was found that a mixed methanotrophic culture is more effective in biodegrading TCE when the second reactor was supplied with a suitable reducing equivalent (e.g. formate) (Mcfarland et al., 1992). Other investigations supported this phenomenon (Aziz et al., 1995; Chang and AlvarezCohen, 1997; Smith and McCarty, 1997; Tschantz et al., 1995). Therefore culture instability can be circumvented since NADH is regenerated from formate oxidation.

To avoid stripping of volatile contaminants, such as TCE, the conversion reactor was usually operated without a gas phase. In the absence of an air feed to replenish the consumed dissolved oxygen, oxygen limitation is possible (Chang and AlvarezCohen, 1997).

In the two-stage systems, microorganisms are often wasted or returned to the growth reactor after treating TCE in the second stage. Wasting the cells after one pass is uneconomical; and a procedure is required to recover microorganisms from residual contaminants in a recycle stream to the growth reactor. In addition, two-stage systems are complicated in configuration and may lead to scale-up problems, which limit their application in bioremediation of TCE.

2.3.3 Immobilized soil bioreactors

The ISBR was first proposed by Karamanev (Karamanev et al., 1997; Karamanev et al., 1998). Soils collected from a site polluted with an organic compound are usually associated with an indigenous microbial consortium capable of degrading it. However, the nutrients, oxygen, humidity and other factors may not be suitable to support the

growth of these bacteria under natural conditions. These requirements can be easily achieved in an ISBR.

The soil particles can be retained in a bioreactor with additional nutrients to enhance the microbial activity. A highly porous geotextile is used to entrap the soil particles. The ISBR is an airlift design containing plates made of semi-permeable geotextile. Air is sparged on one side of the plate resulting in two flow patterns, vertical and horizontal (across the separator). When soil is loaded into the reactor, the water flow across the plate allows the soil to be entrapped in the porous textile supports.

These soil particles not only introduce the microorganisms to the system but also serve as the initial support for biofilm formation. When the soil particles are fixed in the geotextile, the laminar flow across the geotextile prevents friction between particles and maintains a low hydrodynamic shear stress around the soil particles favorable for biofilm development.

The ISBR was successfully used in degradation of pentachlorophenol (PCP). An extremely high volumetric PCP degradation rate (up to 950 mg PCP l^{-1} h^{-1}) was obtained (Karamanev and Samson, 1998), demonstrating its great potential for the degradation of organic contaminants.

The ISBR is also promising in methanotrophic TCE degradation. Type II methanotrophs, (i.e. those expressing sMMO), have a very low specific growth rate, so retention of biomass in the reactor is highly desirable. Soil immobilization is an inexpensive way to accomplish this. It has been suggested that mixed populations are more efficient in TCE degradation because the mixed culture may metabolize the toxic

intermediates generated during TCE degradation (Uchiyama et al., 1992). The 2-l methanotrophic ISBR was reported to have a higher TCE degradation capacity than any other design (Ramsay et al., 2001). A problem with this system was that a small amount of TCE-containing off-gas was produced. A biofilter could be used to remove the TCE. A pilot-scale 2000-l ISBR was also designed and set up. The schematic of this ISBR is shown in Figure 2-5.

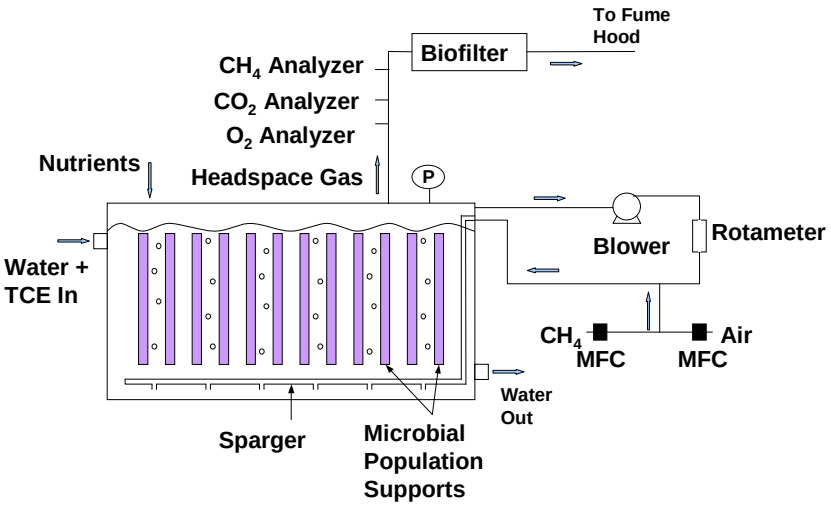

Figure 2-5 Schematic of a 2000-l ISBR (Ramsay et al., 2001).

Table 2-5 Performance of Single-Stage Bioreactors

Reference	Reactor Type	Microbial Culture	$[CH_4]$ (%)	$[TCE]_{in}$ (mg l^{-1})	$[TCE]_{out}$ (mg l^{-1})	Degradation Efficiency (%)	Kinetic Data	Biomass Density	Residence Time (h)	Flow Rate (l min^{-1})	Reactor Volume (l)	Note
Arvin et al., 1991	Rotating cylinder biofilm reactor	Methanotrophs	1.12	0.06-1.2	N/A	23	k_X: (0.86±0.13)×10^{-2} m d^{-1}	1.5 g m^{-2}	4.8	4.7 l d^{-1}	0.95	1
Clapp et al., 1999	Membrane-attached Biofilm reactor	Pure culture of *M. trichosporium* OB3b	25	1.3-4.6	N/A	80-90	N/A	N/A	26	1.25×10^{-4}	0.338	2
Fennell *et al.*, 1993	Expanded -bed	Mixed methanotrophs on diatomaceous earth or granular -activated carbon	Batch 3-4.2 mg l^{-1}*	0.87-13	0.56-8.246	17.1-36.6	*k* 10.6±1.9 mg TCE g VS $^{-1}$.d^{-1} Ks 2.8±1.8 mg TCE l^{-1}	~ 40 g VS l^{-1}				
			Continuous 0.4-1.2 mg l^{-1} *	0.46-42.1	0.24-19.9	24-61	*k*:2.9±0.007 mg TCE g VS $^{-1}$.d^{-1} Ks: 1.5±0.049 mg TCE l^{-1}	7.4-46.7 g VS l^{-1}	1.2-11	N/A	3	3
Fitch *et al.*, 1996	Sequencing biofilm reactor	*M.t.* OB3b** mutant PP358 and RTR on diatomaceous earth pellets or glass beads	1.54-2.77	0.05-0.5	N/A	N/A	k_1: 8-63 l g DW^{-1} d^{-1}	N/A	0.58-1.1	N/A	1.5	4
Phelps et al., 1990	Expanded -bed reactor	Mixed methanotrophs on crushed glass	3	20	N/A	30% over a 5-day period	Degradation rate 1.6 mg $l^{-1}d^{-1}$**	N/A	N/A	N/A	0.72	
Ramsay *et al*, 2001	Immobilized soil bioreactor	Mixed methanotrophs	N/A				k_X			N/A	2	
				2.26	0.061	97.3	39.7 d^{-1}	N/A	21.84			
				18.12	8.46	53.3	42.2 d^{-1}		1.0			

Table 2-5 continued

Reference	Reactor Type	Microbial Culture	$[CH_4]$ (%)	$[TCE]_{in}$ (mg l^{-1})	$[TCE]_{out}$ (mg l^{-1})	Degradation Efficiency (%)	Kinetic Data	Biomass Density	Residence Time (h)	Flow Rate (l min^{-1})	Reactor Volume (l)	Note
Shimomura et al., 1997	Gel-immobilized fluidized bed	*Methylocystis* sp. strain M operated in sequencing mode	Degradation 10% Regeneration 20%	0.9-1.6	0.1-0.2	80-90	k_1 72-144 l (g DW)$^{-1}$ d^{-1}	1.5 g l^{-1}	2.56	N/A	20	5
Speitel Jr. et al., 1993	Packed-bed reactor	Pure culture of *M.t.* OB3b on diatomaceous earth	3.5	0.395	N/A	62.3	k_1 2-7 l g VS^{-1} d^{-1}	10.5 mg VS g DE^{-1}	0.165	0.028	1.77	6
				0.720		20.7	0.9 l g VS^{-1} d^{-1}		0.15	0.031		
Strandberg et al., 1989	Packed-bed reactor	Microbial consortium on ceramic berl saddles	4	0.9	0.2	77.8	k 0.020 min^{-1}	N/A	1.25	0.005	2.16	7
				1.0	0.3	50	0.024 min^{-1}		0.83	0.010		
				1.1	0.5	70	0.016 min^{-1}		0.83	0.010		
				1.0	0.5	50	0.023 min^{1}		0.50	0.020		
				1.1	0.8	27.3	0.020 min^{-1}		0.27	0.035		
				1.0	0.6	40	0.046 min^{-1}		0.18	0.050		

Note:

1. A yellow-brown biofilm was formed on the rotator.
2. This reactor was featured by a counter-diffusion pattern
3. Salmon-colored biomass was observed.
4. Only 1-2 day of appreciable degradation rates were sustained. Not possible to completely restore TCE degradation by switching to growth mode.

5. After 15 days of alternative operation between degradation and reactivation mode, the recovery of TCE degradation activity slowed and k_1 dropped.
6. This bioreactor was used to treat air streams contaminated with chlorinated solvents.
7. The biofilms did not adhere tightly to the packing. The culture appeared to be growing as dense masses in the void spaces between the packing materials (salmon-pink-colored biomass).

* *M.t.* OB3b stands for *Methylosinus trichosporium* OB3b

** Methane was fed to the reactor in dissolved form.

*** Calculated value

k_1: Pseudo-first order rate constant, l g biomass^{-1} d^{-1}

k: Biodegradation rate, g substrate g biomass^{-1} d^{-1}

k_X: Maximum substrate utilization rate

Table 2-6 Comparison of Two-Stage Bioreactors for TCE Degradation

Reference	Microbial Culture	$[CH_4]$ in Growth Phase	$[TCE]_{in}$ (mg l^{-1})	$[TCE]_{out}$ (mg l^{-1})	Degradation Efficiency (%)	Kinetic Data	Biomass Density	Residence Time of Second stage (h)	Reactor Volume (l)		Note
									Growth	Biodegradation	
Aziz *et al.*, 1995	*M.t.* OB3b mutant PP358	N/A	0.09 0.12	N/A	95.3 81.0	k_1 160-900 l g $TSS^{-1}d^{-1}$	200-400 mg TSS l^{-1}	0.24 0.155	20	0.619	1
Chang et al., 1997	Mixed methanotrophs	13%	2.25	1.1 0.1 (with formate)	~50% >90%	k_X: 1.06 d^{-1} 9.59 d^{-1} (with formate)	98 mg VS l^{-1}	1	14	0.6 or 1.2	
McFarland *et al.*, 1992	Mixed methanotrophs	20%	15	N/A	N/A	k: 25.8 mg TCE g VS^{-1} d^{-1}	1.6 g VS l^{-1}	0.67	1.4	0.4	2
Pressman *et al.*, 1999	*M.t.* OB3b* mutant PP358	0.3% Methanol	0.16-1.45	N/A	82-89 61-95**	k_1: 210-2800 l g TSS^{-1} d^{-1}	N/A	5.1 min 1.5-3.7 min	2.5	0.098 0.566	3
Smith and McCarty, 1997	Mixed methanotrophs	13.2%	6.5-8.6	N/A	N/A	k_1: 11-60 l g VSS^{-1} d^{-1} 80-120 l g VSS^{-1} d^{-1} (with formate)	0.1-0.2 g VSS l^{-1}		2.4-7.3	2	
Tschantz *et al.*, 1995	*M.t.* OB3b mutant PP358	N/A	single pass 20	4.37	78	k 109.4 mg TCE g $VS^{-1}d^{-1}$	0.17-0.21 mg VS l^{-1}	4.55	2.4	0.56, 0.8, 0.88, 1.04	4
			cross flow 10	0.853	93	12.84 mg TCE g $VS^{-1}d^{-1}$	0.395 mg VS l^{-1}	4			
U.S. EPA, 1993	*M.t.* OB3b	5%	Bench unit 0.563	63×10^{-3}	89	k_X 2.06 h^{-1}		1.1	1	0.31	
			Pilot unit 1.9	0.25	75		3.6 mg l^{-1}	0.91	300	60	

Note:

1. Hollow fiber membrane bioreactor.

2. The biomass culture had an orange-red color. Formate additions resulted in a rapid increase in TCE degradation.

3. Hollow fiber membrane bioreactor.

4. The second stage consisted of 4 plug-flow contactor columns in series.

* *M.t.* OB3b stands for *Methylosinus trichosporium* OB3b

* * Calculated value

2.3.4 References

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Chapter 3

On-line Estimation of Dissolved Methane Concentration during Methanotrophic Fermentations

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Originally published July, 2006 in

Biotechnology and Bioengineering, 95(5), 788-793

3.1 Abstract

Knowledge of the aqueous phase methane concentration is critical to understanding and controlling process kinetics in methanotrophic bioreactors. Unfortunately since no dissolved methane probe is commercially available, this data must be obtained off-line by the time-consuming gas-liquid partition method. In this paper, we demonstrate how knowledge of the reactor's k_La for oxygen combined with gas phase methane analysis can be used to continuously estimate the aqueous phase concentration of dissolved methane. The on-line estimation was verified in two reactor systems with greatly different values of k_La. In both systems the measured and calculated dissolved methane concentrations were in good agreement although dissolved methane was underestimated in both cases. The utility of this methodology was demonstrated by revealing a possible metabolic bottleneck in the model system.

3.2 Introduction

Methanotrophs are a group of aerobic bacteria that can use methane as their source of carbon and energy and that play important roles in the carbon cycle. Due to their lack of specificity, the soluble methane monooxygenases (sMMOs) produced by some type II methanotrophs can initiate degradation of chlorinated volatile organic compounds (VOCs) and aromatic compounds through cometabolism (Colby et al., 1977; Oldenhuis, et al. 1989; Tsien, et al. 1989). They also possess commercial potential in the production of alkene epoxides, such as propene oxide (Higgins, et al. 1980; Hou, et al. 1980; Hou 1984).

A variety of methanotrophic bioreactors have been developed for VOC biodegradation (Aziz, et al. 1995; Phelps, et al. 1990; Pressman, et al. 1999). One such reactor is the immobilized soil bioreactor (ISBR) which was originally developed as a membrane bioreactor with a self-contained inoculum (soil) for the mineralization of recalcitrant groundwater contaminants such as pentachlorophenol (Karamanev and Samson 1998; Karamanev, et al. 1999). Its application was then expanded to operation as a methanotrophic bioreactor, for the bioremediation of contaminated groundwater (Ramsay, et al. 2001). The kinetics of both VOC degradation and methanotrophic growth is affected by the aqueous phase CH_4 concentration in these systems. However, many methanotrophic biodegradation studies did not include analysis of the aqueous phase methane (Parvatiyar, et al. 1996; Speitel and Leonard 1992; Strandberg, et al. 1989) possibly due to the absence of commercially available dissolved methane probes. Other studies have relied on analyzing dissolved methane by gas chromatography (Arvin 1991; Clapp, et al. 1999; Fennell, et al. 1993; Fitch, et al. 1996, Lanzarone and Mccarty, 1990; Tschantz, et al. 1995) which is time consuming and very difficult to perform on-line. In the present study, we have evaluated the possibility of continuous on-line dissolved methane analysis using a standard infrared methane gas analyzer and a dissolved oxygen (DO) probe.

3.3 Materials and methods

3.3.1 Bioreactors and fermentation conditions

A 1.2 l fermentor (Bioflow II, New Brunswick) was operated as a CSTR at 24℃ and 600 rpm or as an immobilized soil bioreactor (ISBR). Soil was collected from the aerobic-

anaerobic interface of a swampy area near Kingston, Ontario and wet-sieved using a 50 mesh sieve. The soil was incubated under methane-rich conditions and then immobilized in an inert fibrous matrix (polyethylene fiber furniture stuffing, Walmart) contained in a heavy gauge stainless steel screen in the ISBR. A type II methanotroph, isolated from the ISBR mixed culture, was grown in pure culture for the CSTR studies.

The reactor geometry and the gas circulation plumbing for both the ISBR and CSTR configurations are shown in Figure 3-1. The headspace gas was recirculated through each reactor at 0.5 VVM using a diaphragm pump. Since the ISBR had no mechanical mixing, the recirculating gas allowed it to act as an air-lift reactor. During recirculation, the headspace gas passed through an infrared CH_4 analyzer (Guardian Plus methane monitor, Topac Inc. Hingham, MA, USA) and a CO_2 analyzer (S153, Qubit Systems Inc. Kingston, Ontario, Canada). Data acquisition and gas control were performed using a data acquisition board (ADR2000, Ontrak Control Systems Inc. Sudbury, ON, Canada), Labview™ software (National Instruments, Vaudreuil-Dorion, PQ, Canada) and mass flow controllers (FVL2600, Omega, Laval, PQ, Canada) for CH_4 and air (or oxygen enriched air) flow in both CSTR and ISBR modes. Air (or oxygen enriched air) was supplied at a flow rate of 4.88 ml min^{-1}. CH_4 flow rate was varied to maintain the headspace concentration at the set point using Labview™ PID control. During the course of the experiments, DO was always maintained above 20% of saturation. Methane was the sole source of carbon and energy while the mineral salts medium consisted of $NaNO_3$, (0.85 g l^{-1}), KH_2PO_4, (0.53 g l^{-1}), $Na_2HPO_4.7H_2O$, (1.62 g l^{-1}), $MgSO_4.7H_2O$ (37 mg l^{-1}), $FeSO_4.7H_2O$ (11.2 mg l^{-1}), $CaCl_2.2H_2O$ (7 mg l^{-1}), $ZnSO_4.7H_2O$ (0.287 mg l^{-1}), $MnSO_4.7H_2O$ (0.223 mg l^{-1}), H_3BO_3 (0.062 mg l^{-1}), $NaMoO_4.2H_2O$ (0.048 mg

l^{-1}), $CoCl_2.6H_2O$ (0.048 mg l^{-1}), and KI (0.083 mg l^{-1}). The pH was adjusted to 6.88 ± 0.10 using 0.2M H_2SO_4.

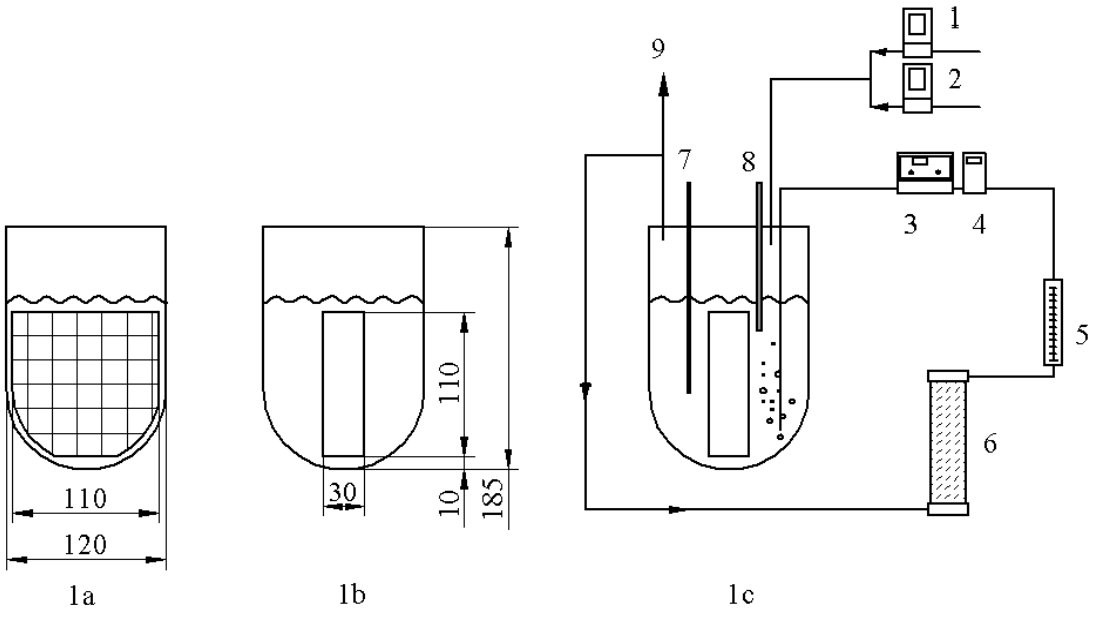

Figure 3-1 Schematic of the 1.2l ISBR. a: Front view. B: Side view. c: Overall schematic. 1 & 2: Mass flow controllers. 3: Infra red methane analyzer. 4: CO_2 analyser. 5: Rotameter. 6: Desiccate column. 7: Temperature sensor. 8: DO probe. 9: Exhaust line.

3.3.2 Analytical techniques

The dry weight of biomass per unit volume of fermentation broth was determined by relating the optical density of samples at 600 nm (Spectronic Unicam UV1, Fisher Scientific, Nepean, ON, Canada) to a calibration curve. Formaldehyde concentrations were determined by a colorimetric method (Chrastil and Wilson, 1975).

3.3.3 Measurement of the dissolved methane concentration

The dissolved methane concentration was measured by gas-liquid phase partition (Fennell, et al. 1992). Triplicate aqueous samples (known volumes of 9-15 ml) were

withdrawn from the reactor with a gas-tight syringe, injected into 25 ml serum bottles containing 0.1 ml of 100 g l^{-1} sodium azide, and immediately sealed with Mininert® valves (Supelco Canada, Mississauga, ON). These bottles were left on a platform shaker at 200 rpm for 1 h. Headspace methane samples (0.1 ml in a 0.25 ml gas-tight syringe) from the serum bottles were then analyzed by gas chromatography (HP5890) with a flame ionization detector (FID) and quantified by relating the peak area to a standard calibration curve and a standard gas (2.46% methane, BOC Canada, Kingston, ON). The dissolved methane concentration was calculated as shown in Equation

$$[CH_4] = \frac{\frac{V_G \cdot P_{CH_4}}{22.4} \cdot M_{CH_4} + V_L \cdot \frac{P_{CH_4}}{H} \cdot M_{CH_4}}{V_L} \qquad 3\text{-}1$$

where V_G is the headspace volume of each serum bottle (ml); P_{CH4} is the methane concentration in the headspace (v/v); M_{CH4} is the molecular weight of methane, H_{CH4} is the Henry's law constant for methane (atm M^{-1}); and V_L is the liquid phase volume of each serum bottle (ml). Since the vast majority of methane has evolved into the headspace, the second part in Equation 3-1 can be neglected.

3.3.4 Estimation of the dissolved methane concentration

Methane uptake rate was estimated online based on a mass balance. At steady state, the methane uptake rate is equal to the rate of methane transfer from the gas to liquid phase.

$$\gamma_{CH_4} = k_L a_{CH_4} \left(\frac{P_{CH_4}}{H_{CH_4}} - [CH_4]_L \right) \qquad 3\text{-}2$$

Thus, the aqueous phase methane concentration can be determined as:

$$[CH_4]_L = \frac{P_{CH_4}}{H_{CH_4}} - \frac{\gamma_{CH_4}}{k_L a_{CH_4}} \quad 3\text{-}3$$

where P_{CH4} is the partial pressure of growth substrate (methane) in the headspace of the reactor (atm), k_La is the mass transfer coefficient (h^{-1}), γ_{CH4} is the methane uptake rate (mg l^{-1} h^{-1}), and H_{CH4} is Henry's law constant for CH_4 (atm mg^{-1} l^{-1}).

3.3.5 Determination of k_La for oxygen and derivation of k_La for methane

The volumetric mass transfer coefficient, k_La, for oxygen in the two reactor configurations was determined by the dynamic method (Tribe, et al. 1995) using a polarographic dissolved oxygen probe (InPro®6800, Mettler-Toledo Process Analytical Inc. MA, USA). If the k_La for O_2 is determined and the ratio of the k_La's for methane and oxygen are known, the dissolved methane concentration can then be determined based on the gas phase methane concentration as in Equation 3-3.

Gas mass transfer coefficients are the product of the liquid phase mass transfer coefficient k_L and specific surface area a. k_L and a are dependent on a number of factors. However, the effect of a may be neglected if only the ratio of k_La between air and methane is considered.

3.3.5.1 Liquid phase mass transfer coefficient k_L

The mass transfer rate of gases with poor solubility in the aqueous phase is essentially determined by resistance across the liquid-film. Under typical fermentation conditions, the k_L of freely rising bubbles has been shown to follow the relationships in Equations 3-4 and 5 (Calderbank and Moo-Young, 1961). For small bubbles (diameter < 2.5mm)

$$k_L = 0.31 D_{gas}^{2/3} \cdot \left(\frac{g\rho_w}{\mu_w} \right)^{1/3} \quad \text{3-4}$$

where μ_w is the dynamic viscosity of the liquid (Pa.s), ρ_w is the density of the liquid, (kg m^{-3}), and D_{gas} is the diffusion coefficient of a gas in the liquid phase ($cm^2\ s^{-1}$).

For larger gas bubbles which do not behave like rigid spheres, k_L was given by

$$k_L = 0.42 \sqrt{D_{gas}} \cdot \left(\frac{\rho_w}{\mu_w} \right)^{1/6} \cdot \sqrt[3]{g} \quad \text{3-5}$$

In the case of mechanical mixing of air-liquid dispersion,

$$k_L = 0.13 \left(\frac{p}{V} \right)^{1/4} \cdot \rho_w^{1/6} D_{gas}^{2/3} \cdot \Big/ \mu_w^{5/12} \quad \text{3-6}$$

where P/V is the volumetric power input (Calderbank and Moo-Young, 1961).

Since mass transfer of different gases is studied in the same reactor and operating conditions, μ_w and ρ_w can be ignored and a linear relationship obtained.

$k_L a \propto \sqrt{D_{gas}}$ for freely rising bubbles 3-7

or $k_L a \propto D_{gas}^{2/3}$ for a mechanically mixed system. 3-8

3.3.5.2 Estimation of diffusivity

Diffusivity of most gases may be found in the literature or, if not available, they can be estimated by the Wilke-Chang correlation (Wilke and Chang, 1955):

$$D_{gas} = 7.4 \times 10^{-8} \frac{T(x_a M)^{1/2}}{\mu_w V_m^{0.6}} \quad \text{3-9}$$

where M is the solvent molecular weight, V_m is the molecular volume of solute at its boiling point, ($cm^3\ g^{-1}\ mol^{-1}$), μ_w is the viscosity of the solution in centipoises, and T is absolute temperature in K. The parameter x_a is 2.6 for aqueous systems (Wilke and Chang, 1955). Since liquid density, viscosity, and temperature are all the same, Equations 3-7 and 3-8 respectively are reduced to:

$$k_L a \propto \left(1/V_m \right)^{0.3} \text{ for an ISBR, or} \qquad 3\text{-}10$$

$$k_L a \propto \left(1/V_m \right)^{0.4} \text{ for a mechanically mixed system} \qquad 3\text{-}11$$

3.3.5.3 Relation of k_La for methane and air

Since V_m for oxygen and methane are 25.6 (Welty, et al. 1976) and 37.9 (Poling, et al. 2001) respectively, the following relation between the mass transfer coefficients of methane and oxygen can be obtained.

In an ISBR,

$$k_L a_{O_2} = 1.125 k_L a_{CH_4} \qquad 3\text{-}12$$

In a mechanically mixed system,

$$k_L a_{O_2} = 1.169 k_L a_{CH_4} \qquad 3\text{-}13$$

3.4 Results

We wished to demonstrate that the aqueous phase methane concentration could be reliably determined with Equation 3-3 using data for the k_La of oxygen and the correlations shown in Equations 3-12 and 3-13. Since this method is dependent on k_La

data, the methodology was tested in two bioreactor systems with very different k_La values. The k_La for oxygen in the ISBR was determined to be 1.24 h^{-1} with a standard deviation of 0.12 h^{-1}. The k_La for oxygen in the CSTR was 19.05 h^{-1} with a standard deviation of 0.79 h^{-1}. Thus, based on Equations 3-11 and 3-12, the k_La for methane should be 1.10 h^{-1} in the ISBR and 16.30 h^{-1} in the CSTR.

Headspace methane concentrations (up to 25% v/v) were controlled in the ISBR and in the CSTR when the reactors were operated in continuous mode at a dilution rate of 0.05 h^{-1}. The culture was not allowed to reach steady state but the gas phase CH_4 concentration was well controlled at the desired value. At each headspace CH_4 concentration, the aqueous phase CH_4 concentration was calculated based on gas phase methane and the k_La for methane. The results were compared to measured CH_4 concentration values in aqueous phase samples taken at the same time and analyzed by headspace gas chromatography (Figure 3-2 and Figure 3-3). The correlation coefficient between calculated and measured concentrations was 0.917 for the ISBR and 0.956 for the CSTR. The R^2 was 0.95 in both cases. Thus, there was good agreement between the measured and calculated dissolved methane concentration in both reactor configurations but the measured values were always lower than the calculated ones probably due to loss of methane during sampling.

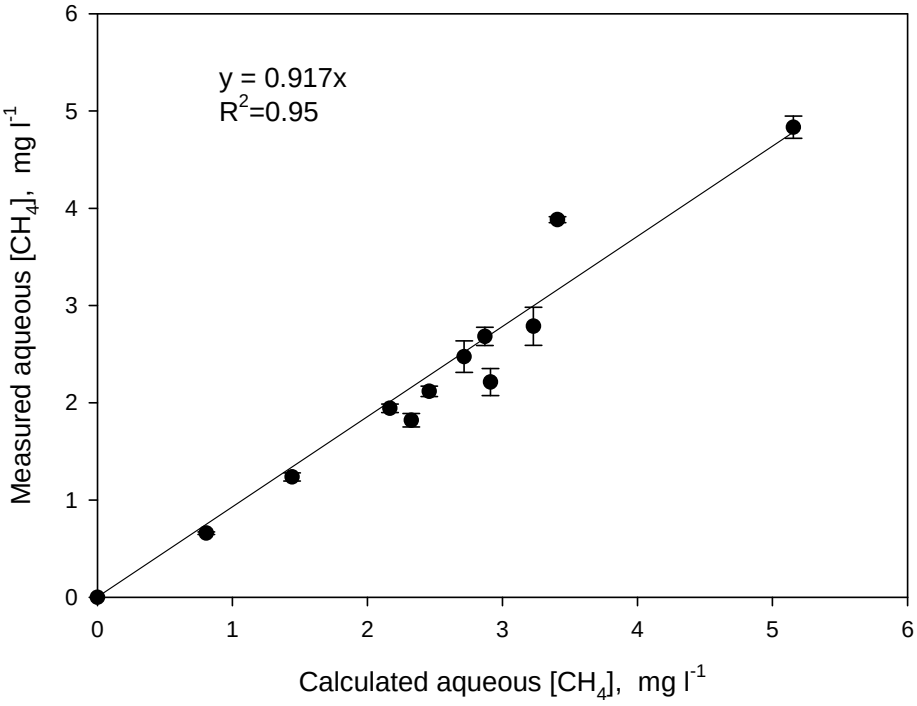

Figure 3-2 Comparison of aqueous phase methane concentration obtained experimentally and by calculation in the 1.2 l ISBR during continuous flow study.

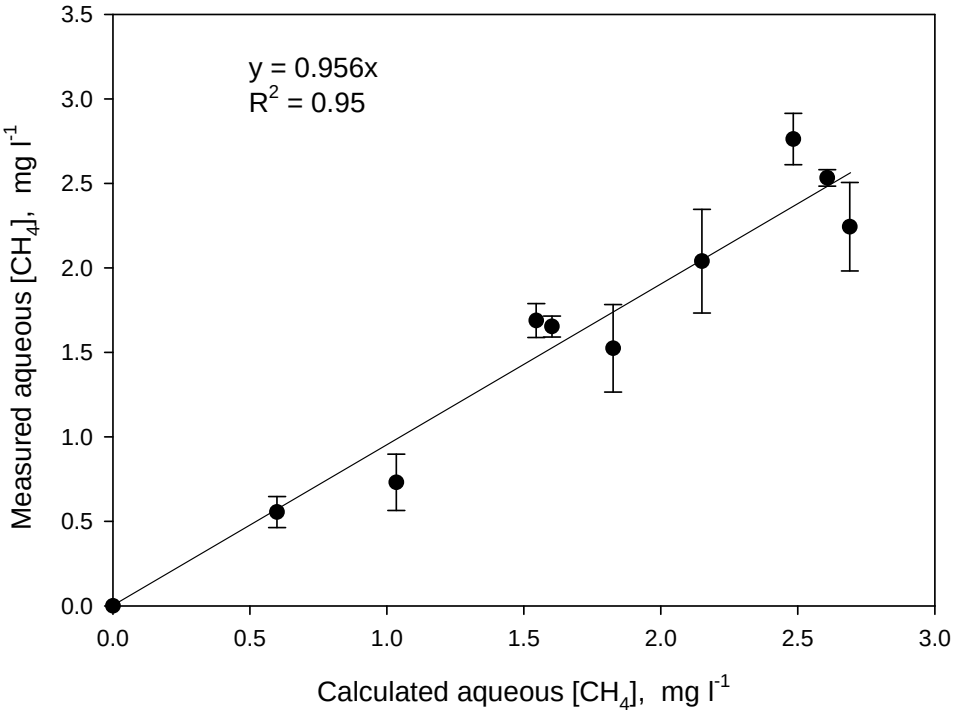

Figure 3-3 Comparison of aqueous phase methane concentration obtained experimentally and by calculation in the 1.2 l CSTR during continuous flow study.

A study was conducted to determine the effect of the gas phase CH_4 concentration on an axenic methanotrophic culture when the reactor was operated in CSTR mode as a chemostat at a constant dilution rate (0.05 h^{-1}). The headspace methane concentration was varied and dissolved methane concentrations were measured at steady state. The steady state methane uptake rates were plotted against the mass transfer driving force based on the measured gas phase CH_4 concentration and the measured (i.e. not the calculated) aqueous phase CH_4 concentration (Figure 3-4) as in Equation 3-3 which is another form of Equation 3-2. The slope is the measured k_La for methane. It is 15.96 h^{-1} with an R^2 of 0.906, a very similar value to the previously calculated k_La (16.30 h^{-1}) that had been based on the correlation in Equation 3-2 and on the measured k_La for oxygen.

The CH_4 uptake rate (as well as the biomass concentration and CO_2 production rate all increased in a linear fashion directly proportional to the increase in gas phase methane concentration. This demonstrates that CH_4 is the limiting nutrient in terms of the amount of growth. However, since the dilution rate was held constant, chemostat theory dictates that the growth rate limiting substrate should also remain at a constant concentration, but in this case the dissolved methane concentration increased as the gas phase methane concentration was increased. This behavior may have been caused by a metabolic bottleneck. The concentration of formaldehyde, a metabolite of CH_4 (metabolic pathway shown in Figure 3-5), was found to remain constant as the aqueous phase methane concentration increased (Figure 3-4, open circles), indicating that its uptake limits the growth rate of this particular organism.

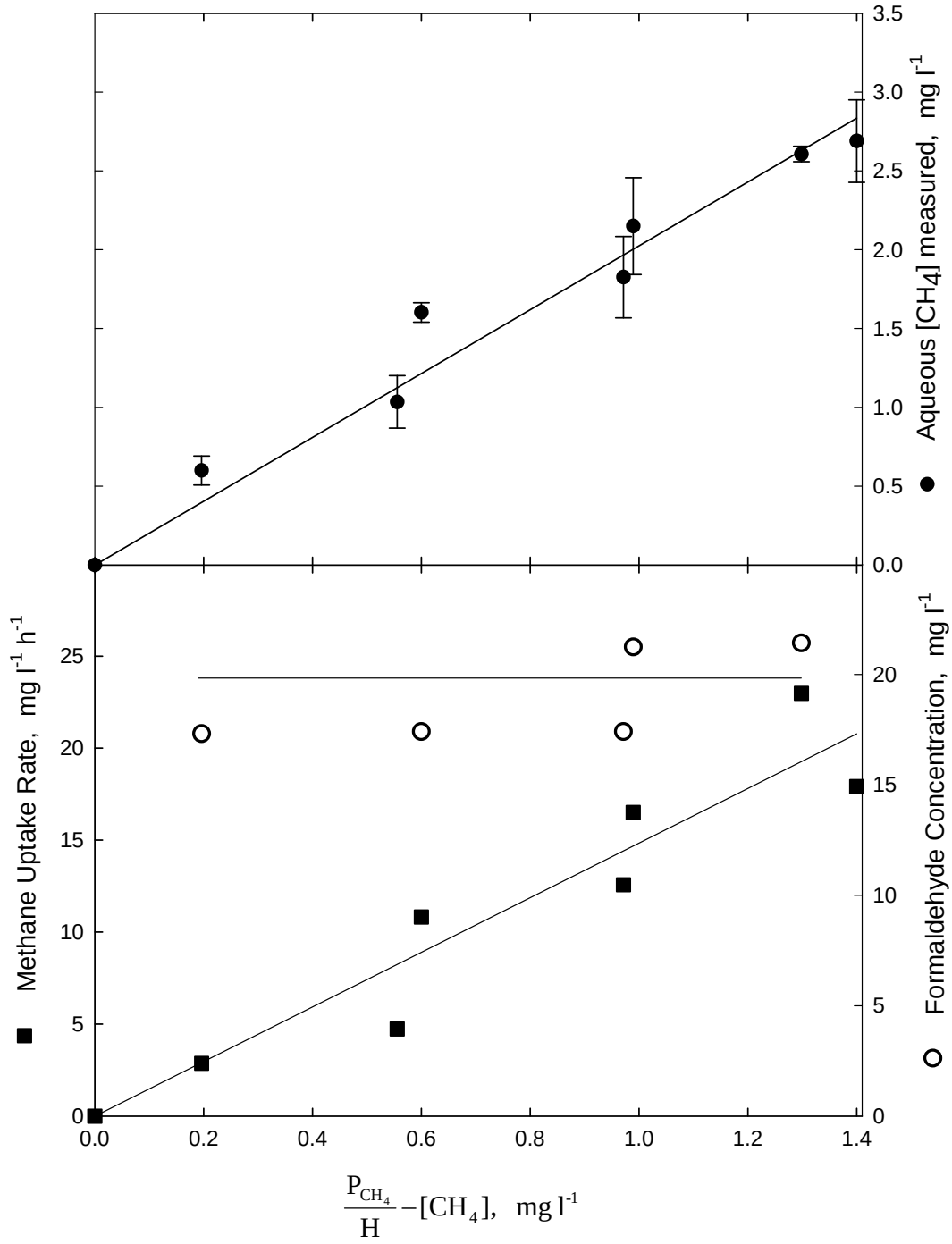

Figure 3-4 Dependence of MUR, formaldehyde concentration and dissolved methane concentration on mass transfer driving force, $PCH_4/H - [CH_4]$, during a continuous flow study of a type II methanotroph. Dilution rate: 0.05 h^{-1}. Temperature: 24°C. Agitation: 600 rpm.

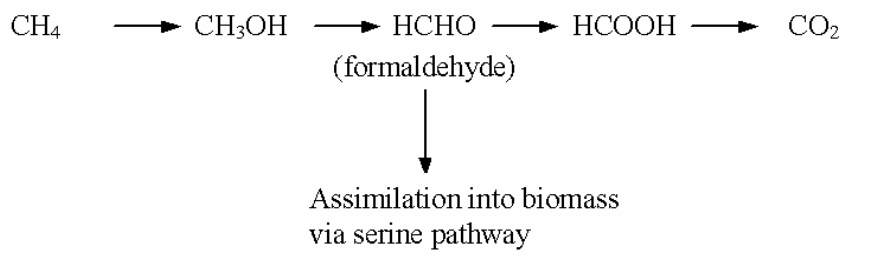

Figure 3-5 Metabolic pathway for methane oxidation (Hanson and Hanson, 1996).

3.5 Discussion

As shown in Equation 3-3, this methodology relies on accurate knowledge of the k_La and the methane uptake rate. If the gas flow rates are known and if the gas phase methane concentration is monitored, the methane uptake rate can be accurately determined online as was the case in our study. The accuracy of this method also depends on the k_La which must be evaluated for each reactor system and operating condition. As demonstrated in Equations 3-6 and 3-9, if temperature, mixing and aeration rate remain constant, only viscosity will affect k_La. Therefore k_La should remain relatively constant from batch to batch and during an individual fermentation, especially in methanotrophic cultures where cell density is typically very low. However, if there is significant change in the k_La, due to extracellular polysaccharide production for example, automated k_La determinations can be performed via control of gas flow with on-line dissolved oxygen data as in Kamen, et al. (1996).

Although the results indicated that, in this case, CH_4 was not the growth-rate-limiting substrate, knowledge of its concentration is often critical to understanding

methanotroph growth kinetics. As an example, stimulation of methanotroph activity is frequently used for *in-situ* bioremediation of groundwater contaminated with halogenated solvents such as trichloroethylene (Sutfin 1996; Sutfin and Ramey 1997; Travis and Rosenberg 1997). Since methanotroph proliferation is desired but methane competes with the solvents for the methane monooxygenase active site (Alvarez-Cohen and McCarty 1991; Anderson and McCarthy 1996; Speitel, et al. 1993), knowledge of the kinetics is essential for process modeling.

This approach in the determination of the dissolved gas concentration can also be applied to other biological processes where dissolved gas concentration cannot be obtained online using a probe.

Acknowledgements

This research was supported by the Natural Science and Engineering Research Council of Canada. Y. Yu was the recipient of an Ontario Graduate Scholarship, McLaughlin Scholarship,and Queen's Graduate Awards.

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Chapter 4

Production of Soluble Methane Monooxygenase during Growth of *Methylosinus trichosporium* on Methanol

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Accepted by Journal of Biotechnology on Sep 23, 2008

4.1 Abstract

Soluble methane monooxygenase (sMMO) can degrade many chlorinated and aromatic pollutants. It is produced by certain methanotrophs such as *Methylosinus trichosporium* when grown on methane under copper limitation but, due to its low aqueous solubility, methane cannot support dense biomass growth. Since it is water soluble, methanol may be a more attractive growth substrate, but it is widely believed that sMMO is not produced on methanol. In this study, when the growth-limiting substrate was switched from methane to methanol, in the presence of the particulate MMO inhibitor, allylthiourea, growth of *M. trichosporium* OB3b continued unabated and sMMO activity was completely retained. When allylthiourea was then removed, sMMO activity was maintained for an additional 24 generations, albeit at a slightly lower level due to the presence of 0.70 μM of Cu^{2+} in the feed medium. While a biomass density of only 2 g l^{-1} could be obtained on methane, 7.4 g l^{-1} was achieved by feeding methanol exponentially, and 29 g l^{-1} was obtained using a modified feeding strategy employing on-line carbon dioxide production measurement. It was concluded that methanol can be employed to produce large amounts of *M. trichosporium* biomass containing sMMO.

4.2 Introduction

Under copper limitation, type II methanotrophs, the type X methanotroph, *Methylococcus capsulatus* Bath, and some type I methanotrophs can produce soluble methane monooxygenase (sMMO) rather than the usual copper-containing particulate type (pMMO) (Hanson and Hanson, 1996; Koh et al., 1993). The soluble form of MMO has a broad substrate range and can degrade many chlorinated and aromatic organic

compounds (Colby et al., 1977; Oldenhuis et al., 1989; Tsien et al., 1989). For this reason, methanotrophic bioreactors with high sMMO activities were developed to treat contaminated groundwater (Aziz et al., 1995; Phelps et al., 1990; Pressman et al., 1999), to produce sMMO-containing methanotrophs for the bioaugmentation of contaminated sites (Duba et al., 1996; Rockne et al., 1998; Shah et al., 1996) or for industrial biotransformations (Lee et al., 1996). Productivities in such bioreactors are dependent on the amount of sMMO-containing biomass. However, to our knowledge, the highest dry biomass concentration ever attained without biomass recycle by a culture using methane as the sole source of carbon and energy was about 5 g l^{-1} (Asenjo and Suk, 1986) in a baffled shake-flask and about 18 g l^{-1} (with low sMMO activity) in a bioreactor (Shah et al., 1996). Higher biomass concentrations must be obtained to be economically attractive.

The aforementioned limitations in bioreactor productivity were at least partly due to the low aqueous solubility of methane. As a carbon source, methane is first oxidized to methanol by MMO. Many methanotrophs are able to grow on methanol, with no need for methane. Methanol is inexpensive and presents fewer safety concerns than methane. More importantly, methanol is completely miscible in water so there is no mass transfer limitation of growth. Methylotrophs have been produced in high-density culture (Bourque et al., 1995), but none of these produce sMMO. *Methylosinus trichosporium* OB3b (ATCC 35070) is the type II methanotroph most frequently studied for sMMO production. It has been grown to a biomass concentration of 3.2 g l^{-1} in chemostat culture on 1.0% methanol (v/v) (Best and Higgins, 1981). A sMMO constitutive mutant of *M. trichosporium* OB3b (PP358) produced 1.0 g l^{-1} biomass on methanol (Fitch et al., 1996). Both aforementioned cultures retained sMMO activity on methanol. However, many

publications have reported, and it is still generally believed, that no sMMO production occurs in type II methanotrophs grown on methanol (Hou et al., 1979; Patel et al., 1980; Sullivan et al., 1998). Based on this belief, significant studies have been conducted on the optimization and scale-up of *M. trichosporium* OB3b fermentations employing methane (Carmen et al., 1996). In this study, we evaluated the efficacy of producing sMMO during growth on methanol and the feasibility of producing dense cultures of *M. trichosporium* OB3b using methanol the sole source of carbon and energy.

4.3 Material and methods

4.3.1 Organism and growth medium

Methylosinus trichosporium OB3b was maintained on nitrate mineral salts Noble agar plates and incubated in a methane-rich atmosphere (20% CH_4 v/v). Two media were used for cultivation. Nitrate mineral salts (NMS) medium consisted of (in g l^{-1}) $NaNO_3$, 0.85; KH_2PO_4, 0.53; $Na_2HPO_4.7H_2O$, 1.62; $MgSO_4.7H_2O$, 0.037; $FeSO_4.7H_2O$, 0.0112; $CaCl_2.2H_2O$, 0.007; and 1 ml l^{-1} of trace element solution containing (in g l^{-1}) $ZnSO_4.7H_2O$, 0.287; $MnSO_4.7H_2O$, 0.223; H_3BO_3, 0.062; $NaMoO_4.2H_2O$, 0.048; $CoCl_2.6H_2O$, 0.048; and KI, 0.083. The pH was adjusted to 6.88 ± 0.10 using 0.2 M H_2SO_4. When grown on methanol, a variation of Choi's medium (Bourque et al., 1995) was used. It contained (in g l^{-1}): $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 1.305; $Na_2HPO_4.7H_2O$, 4.02; $MgSO_4.7H_2O$, 0.45; $CaCl_2.2H_2O$, 0.0033; $FeSO_4.7H_2O$, 0.0013; and 1 ml l^{-1} of the trace element solution described above. All media were prepared with de-ionized water. Stock solutions containing 10 g l^{-1} $CaCl_2.2H_2O$ or $FeSO_4.7H_2O$ were also prepared.

4.3.2 Inoculum preparation

Erlenmeyer flasks (250 ml) containing 50 ml medium were autoclaved. Colonies of *M. trichosporium* OB3b maintained on noble agar plates were transferred to the medium aseptically. For methanol-grown cultures, 10 g l^{-1} methanol was introduced aseptically. The flasks were covered with foam stoppers and incubated at 30°C and 200 rpm on a gyro-rotary shaker (Innova 44, New Brunswick Scientific, USA). For methane-grown innocula, the flasks were sealed with rubber stoppers fitted with two 0.2 μM filters. After inoculation, a methane/air mixture (20% CH_4 v/v) was introduced to the flasks through the filter for 5 min at a flow rate of 3 l min^{-1} and then incubated at 30°C and 200 rpm. When growth was observed, this culture was transferred to 2-l Erlenmeyer flasks containing 200 ml NMS medium to prepare inocula for reactor fermentations. Gas feed and incubation conditions were the same as above.

4.3.3 Bioreactors and fermentation conditions

Fed-batch fermentations were performed in a 2-l stirred-tank bioreactor (Multigen F-2000, New Brunswick Scientific, NJ, USA) with 1.25 l Choi's medium and 250 ml of a 72-h innoculum at 30°C, 500 rpm agitation, and 0.5 l min^{-1} air or an air and oxygen mixture. Headspace CO_2 was analyzed by a Guardian infrared CO_2 analyzer (Topac Inc. Hingham, MA, USA). pH was controlled at 6.88 by the addition of 14% (w/v) ammonium hydroxide using a Bioengineering AG (Wald, Switzerland) controller. Data acquisition, substrate feeding, and DO control were performed using a data acquisition board (ADR2000, Ontrak Control Systems Inc. Sudbury, ON, Canada), and a Labview™ data acquisition and control program (National Instruments, Vaudreuil-Dorion, PQ, Canada). DO was maintained at 40% or more of air saturation using the Labview™ PID

to adjust mass flow controllers and balance the air and oxygen flow rates as described previously (Sun et al., 2006). Substrate feeding was automated using a peristaltic pump (Masterflex® 7520-35, Cole-Parmer International Inc., Chicago, IL, USA) by comparing the predicted substrate feeding and the amount of substrate fed as measured by a balance (P/PI-4002, Denver Instruments, Denver, Colorado).

The study of sMMO activity in different growth media was conducted in a 1.2 l Bioflo IIc reactor (New Brunswick Scientific, NJ, USA) operated as a chemostat at a dilution rate of 0.05 h^{-1}. When the culture was grown on methane, headspace methane content was maintained at 22% as described in Yu et al. (2006). Headspace methane content was continuously analyzed by recirculating headspace gas through an infrared CH_4 analyzer (Guardian Plus methane monitor, Topac, Inc., Hingham, MA). Temperature was controlled at 30°C and agitation at 700 rpm.

4.3.4 Substrate feeding strategy

Fed-batch fermentations began in batch mode with 5 g l^{-1} of methanol. At 72 h, exponential substrate feeding was initiated according to Equation 4-1.

$$S_t = \frac{X_t}{Y_{X/S}} = \frac{X_0}{Y_{X/S}} \cdot e^{\mu t} \qquad 4\text{-}1$$

where S_t is the total amount of methanol to be fed at time t (g), X_t is the desired biomass at time t (g), X_o is the biomass when exponential feeding began (g), μ is the desired growth rate (h^{-1}), and $Y_{X/S}$ is the expected yield of biomass from substrate (g biomass g substrate^{-1}).

The feeding strategy described in Equation 4-1 was modified based on carbon dioxide production for better control as shown in Equation 4-2.

$$S_t = \frac{CCP_t}{Y_{CO_2/S}} \qquad 4\text{-}2$$

where CCP_t is the cumulative carbon dioxide production (g), and $Y_{CO_2/S}$ is the yield of carbon dioxide from substrate (g g^{-1}). This strategy has been shown to be effective in the high-density biomass cultivation of *Pseudomonas putida* (Sun et al., 2006).

4.3.5 Calculation of specific growth rates

Specific growth rates were calculated based on carbon dioxide production rate (CPR) as shown in Equation 4-3:

$$\mu_{CPR} = \frac{dCPR/dt}{CPR} \qquad 4\text{-}3$$

where μ_{CPR} is specific growth rate based on carbon dioxide (h^{-1}), CPR is the carbon dioxide production rate (g h^{-1}), and t is time (h).

4.3.6 Analytical techniques

4.3.6.1 Biomass

The dry weight of biomass per unit volume of fermentation broth was determined by relating the optical density of samples at 600 nm (Spectronic Unicam UV1, Fisher Scientific, Nepean, ON, Canada) to a calibration curve.

4.3.6.2 Copper

Aqueous samples from the reactor feed or effluent were collected and centrifuged at 8,000 x*g* to remove biomass or precipitates. The supernatants were stored in glass tubes pre-washed with 0.2 M sulfuric acid and de-ionized water. Copper content was assayed by inductively coupled plasma-optical emission spectrometer (ICP-OES, Varian, AX-Vista Pro CCD).

4.3.6.3 Formaldehyde

Samples from the reactor were centrifuged at 12,500 ×*g* for 6 min. Formaldehyde concentrations in the supernatants were determined by a colorimetric method (Chrastil and Wilson, 1975).

4.3.6.4 sMMO activity

sMMO activity was determined in triplicate using the colorimetric method of Brusseau and Tsien (Brusseau et al., 1990; Tsien et al., 1989). Cell cultures were centrifuged and resuspended in NMS medium to a biomass concentration of 0.5 g l^{-1}. Concentrated potassium formate was added to a final concentration of 20 mM. The cell suspensions were incubated with naphthalene crystals at 200 rpm, and 30°C for 1 h. The reaction solutions were then centrifuged and the naphthol content was determined by adding 0.1 ml freshly prepared 1% o-dianisidine tetrazotized dye (Sigma-Aldrich) to 1 ml supernatant at the appropriate dilution. A purple color indicates sMMO activity. After mixing, 0.4 ml acetic acid was added to stabilize the color. The absorbance at 530 nm was measured immediately and related to a standard curve (0-7 mg naphthol l^{-1}).

Specific sMMO activity was calculated as naphthalene oxidation rate per gram of biomass.

4.3.6.5 Measurement of headspace and aqueous phase methanol concentration

Aqueous samples were withdrawn from the reactor and centrifuged at 12,500 ×*g* for 6 min. Methanol concentration in the supernatant was analyzed by a gas chromatograph (Varian 3400, Varian Canada Inc., Mississauga, ON) equipped with a Carbowax®-PEG column and a flame ionization detector (FID). Butanol was used as the internal standard. Injector and detector temperatures were 120°C and 250°C respectively. Helium was used as the carrier gas at a flow rate of 5 ml min^{-1}. The column temperature was held at 60°C for 1 min, increased to 150°C at 20°C min^{-1}, and then held for 1 min. Methanol concentration was determined by relating peak area to that of a standard and corrected with the internal standard. For more rapid methanol analysis, 200 µl bioreactor off-gas samples were withdrawn from the reactor exhaust line with a gas-tight syringe and then injected in a Hewlett-Packard 5890 GC with a FID detector and a HP-5 column. Injector temperature, detector temperature, and helium flow rate were the same as the aqueous sample analysis but the oven temperature was maintained at 90°C. Headspace samples were compared with a methanol standard. Aqueous methanol concentration was then estimated using a Henry's law constant of 1.4×10^2 M atm^{-1} (Yaws and Yang, 1992).

4.4 Results

4.4.1 sMMO activity

The capacity of *Methylosinus trichosporium* OB3b to retain sMMO activity while growing on methanol was investigated. The culture was initially grown in batch culture on NMS

medium with methane as the sole source of carbon and energy. Although the medium was formulated to minimize copper content, about 0.70 μM Cu^{2+} was still present in the feed. Therefore, allylthiourea (15 μM) was added to inhibit possible synthesis of pMMO. Continuous medium flow began at 71 h with a dilution rate of 0.05 h^{-1}. Headspace methane was maintained at 22% v/v. As steady state (defined as less than 5% of variation over one volume change) was approached (judged by methane uptake rate, CO_2 production, biomass concentration, and sMMO activity), sMMO activity of about 550 μmol naphthol produced g $biomass^{-1}$ h^{-1} was obtained (Figure 4-1), which is comparable to the sMMO activity usually reported for this strain when grown on methane in the absence of copper (Bowman and Sayler, 1994; Koh et al., 1993). After stopping the methane supply and switching to the feed containing methanol, the majority of the sMMO activity remained intact. During the brief period when both methane and methanol were present, higher sMMO activity was observed.

After 6 generations of growth on methanol with allylthiourea, the feed was changed to one without allylthiourea. The specific sMMO activity dropped only slightly to 490 μmol naphthol produced g $biomass^{-1}$ h^{-1} (Figure 4-2). This level of sMMO activity was maintained for 24 generations. Subsequent addition of 4.5 μM copper to the growth medium caused sMMO activity to diminish to less than 10 μmol naphthol produced g $biomass^{-1}$ h^{-1}.

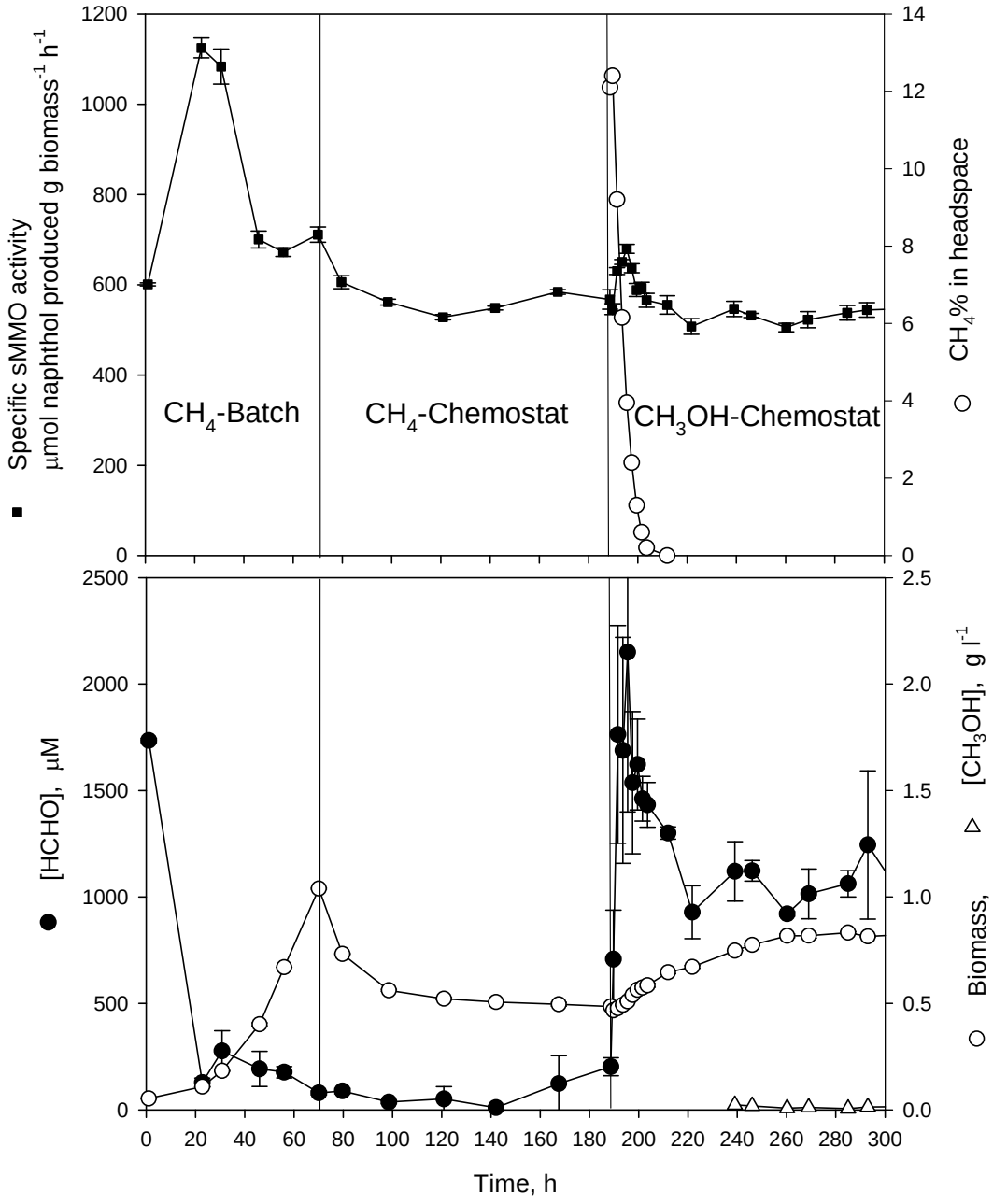

Figure 4-1 Batch and chemostat growth of *Methylosinus trichosporium* OB3b. The culture was first grown in batch culture on methane with 15 µM of the pMMO inhibitor allylthiourea. Continuous flow of the same medium began at 71 h at a dilution rate of 0.05 h^{-1}. After steady state was achieved on methane (190 h), the methane supply was stopped and a medium containing 4 g l^{-1} methanol and 15 µM allylthiourea was continuously fed.

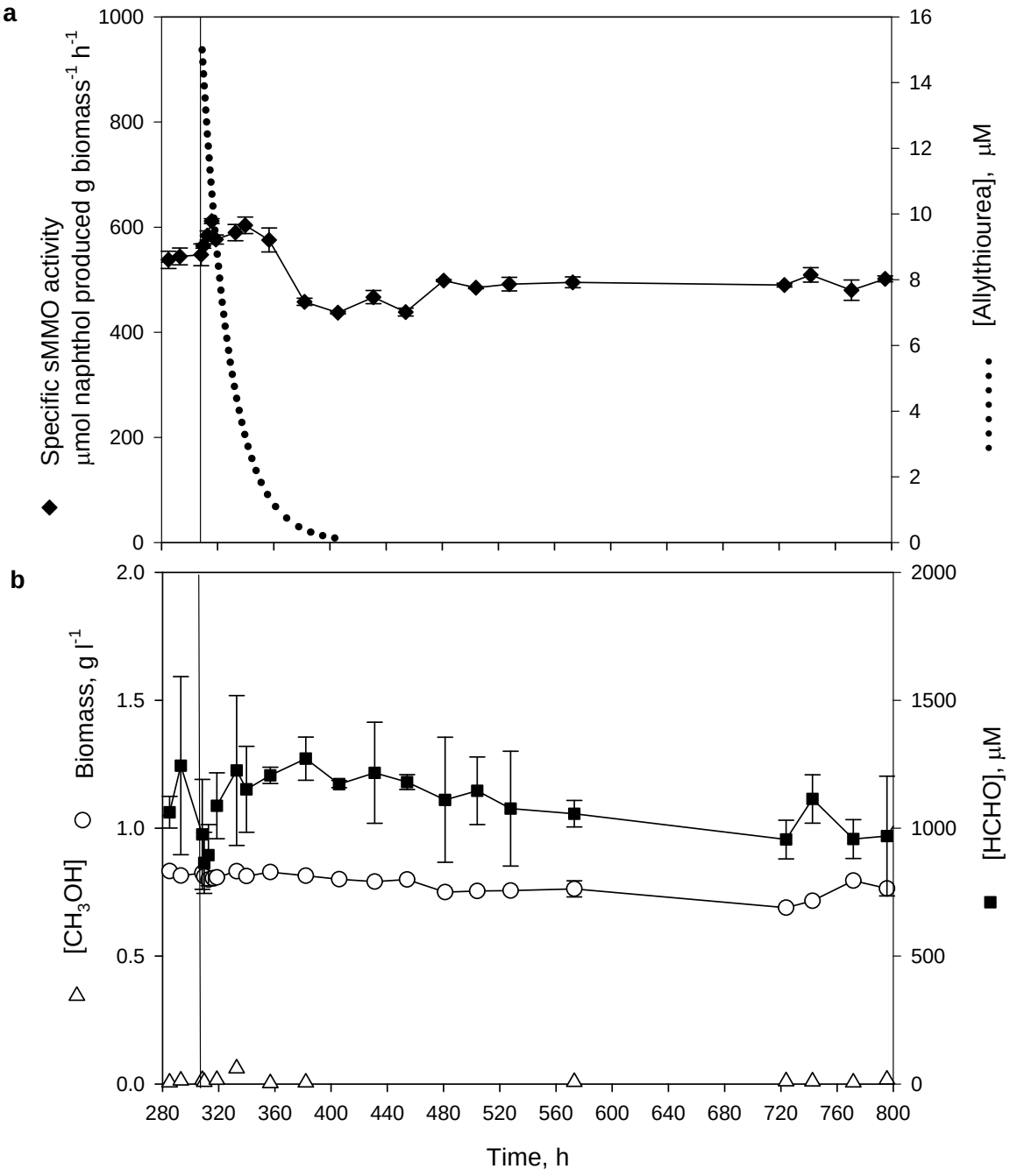

Figure 4-2 Chemostat culture of *Methylosinus trichosporium* OB3b without allylthiourea. The culture depicted in Figure 4-1 was continued, but at 309 h, allylthiourea was omitted from the medium. This resulted in only a small decrease in sMMO activity. The dotted line shows the expected concentration of allylthiourea remaining in reactor due to dilution.

4.4.2 Fed-batch fermentation using exponential feeding of methanol

Once stability of sMMO in methanol-grown cells was established, a methodology for producing high-density *M. trichosporium* OB3b biomass was investigated. Growth in shake-flasks occurred at up to 40 g l^{-1} of initial methanol concentration but only after a long lag period, so batch cultivation was initiated with 5 g l^{-1} of methanol (Figure 4-3). At 72 h, methanol feeding began at a rate designed to produce a μ of 0.06 h^{-1}. However the μ_{CPR} (specific growth rate calculated based on CO_2 production) was only 0.037 h^{-1}, this resulted in methanol accumulation in the fermentation broth. At 86.2, 109.4, 135.8, and 157.5 h, methanol feeding was stopped for 6.5, 3.8, 12.2 and 14.0 h respectively (as seen in the plateaus in the "methanol fed" data in Figure 4-3) to allow the concentration to decrease. During fed-batch fermentation, mineral salts (Ma, Fe, Ca) were supplemented based on the yield of biomass from these metals, which were determined in a previous fermentation to be 485 for $Y_{Mg/X}$, 1947 for $Y_{Fe/X}$, 2482 for $Y_{Ca/X}$ (g biomass g^{-1} metal). Over 160 h of fed-batch fermentation, 7.4 g l^{-1} biomass was produced.

The inhibitory effect of methanol was examined at time periods where changes in growth rate and methanol concentration occurred. For example, at 157.5 h, after methanol feeding was stopped, the residual methanol dropped from 3.28 to 0.47 g l^{-1}, μ_{CPR} increased to as high as 0.3 h^{-1} (Figure 4-3). The results demonstrated that a methanol concentration above 3 g l^{-1} has a significant inhibitory effect on metabolic activity. The increase in μ_{CPR} caused the methanol concentration to drop rapidly to growth-limiting values (less than 0.47 g l^{-1}). This caused the μ_{CPR} to decrease to a point where cell growth could not be recovered. These results are typical of several fermentations conducted in a similar manner.

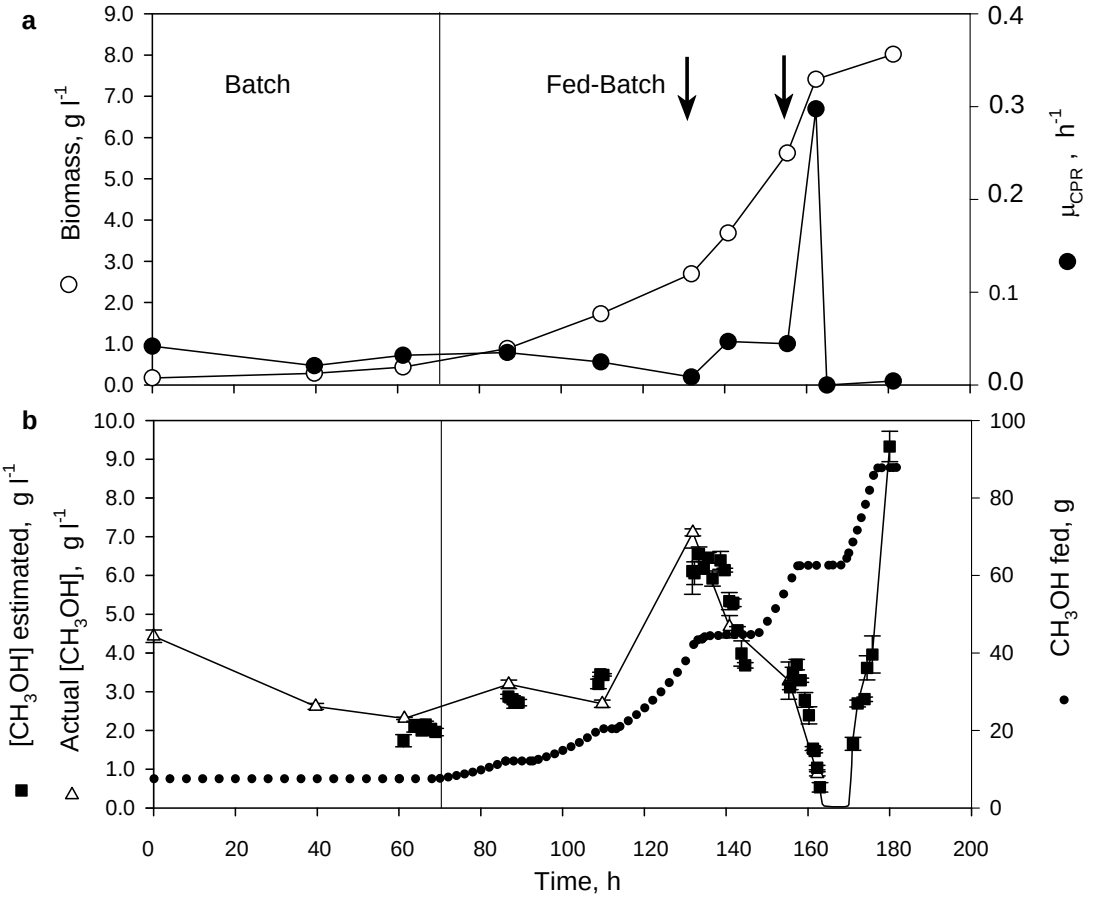

Figure 4-3 Fed-batch fermentation of *Methylosinus trichosporium* OB3b on methanol using an exponential feeding strategy. Methanol feeding was designed to obtain a μ of 0.06 h^{-1}. Methanol concentration in the aqueous phase was measured in liquid samples (△, actual) or estimated from headspace samples (■, estimated). At 131.8 h (first arrow), 1 ml each of the $CaCl_2.2H_2O$, $FeSO_4.7H_2O$, and trace element stock solutions was added. At 155.3 h (second arrow), 2 ml of each of these solutions was added.

This ineffective feeding strategy was then modified. Instead of using a pre-set μ, CCP was used to control methanol feeding. $Y_{CO2/S}$ was determined from previous experiments to be 0.73 g g^{-1} and two fermentations with two different methanol concentration set-points were performed to assess the new feeding strategy (Figure 4-4).

Methanol concentration was better controlled at low biomass concentrations, but high methanol demand (based on CCP) at higher biomass concentrations resulted in over-feeding in both fermentations. A dry weight biomass density of 19 g l^{-1} was obtained in 169 h when the methanol concentration was controlled at about 3 g l^{-1} (Figure 4-4a). An even higher biomass density of 29 g l^{-1} was achieved in only 119 h by further lowering the methanol concentration set-point to about 2 g l^{-1} (Figure 4-4b). Whether due to deviation of the CO_2 analyzer or an actual change of cell yield, the measured $Y_{CO2/S}$ was not constant throughout the fermentation. Slight adjustments of the yield value were necessary. The $Y_{CO2/S}$ used in the control equation (Equation 4-2) was gradually increased to 0.87 by the end of both fermentations. For all fed-batch fermentations, mineral salts were supplemented at points indicated by arrows in Figure 4-3 and Figure 4-4.

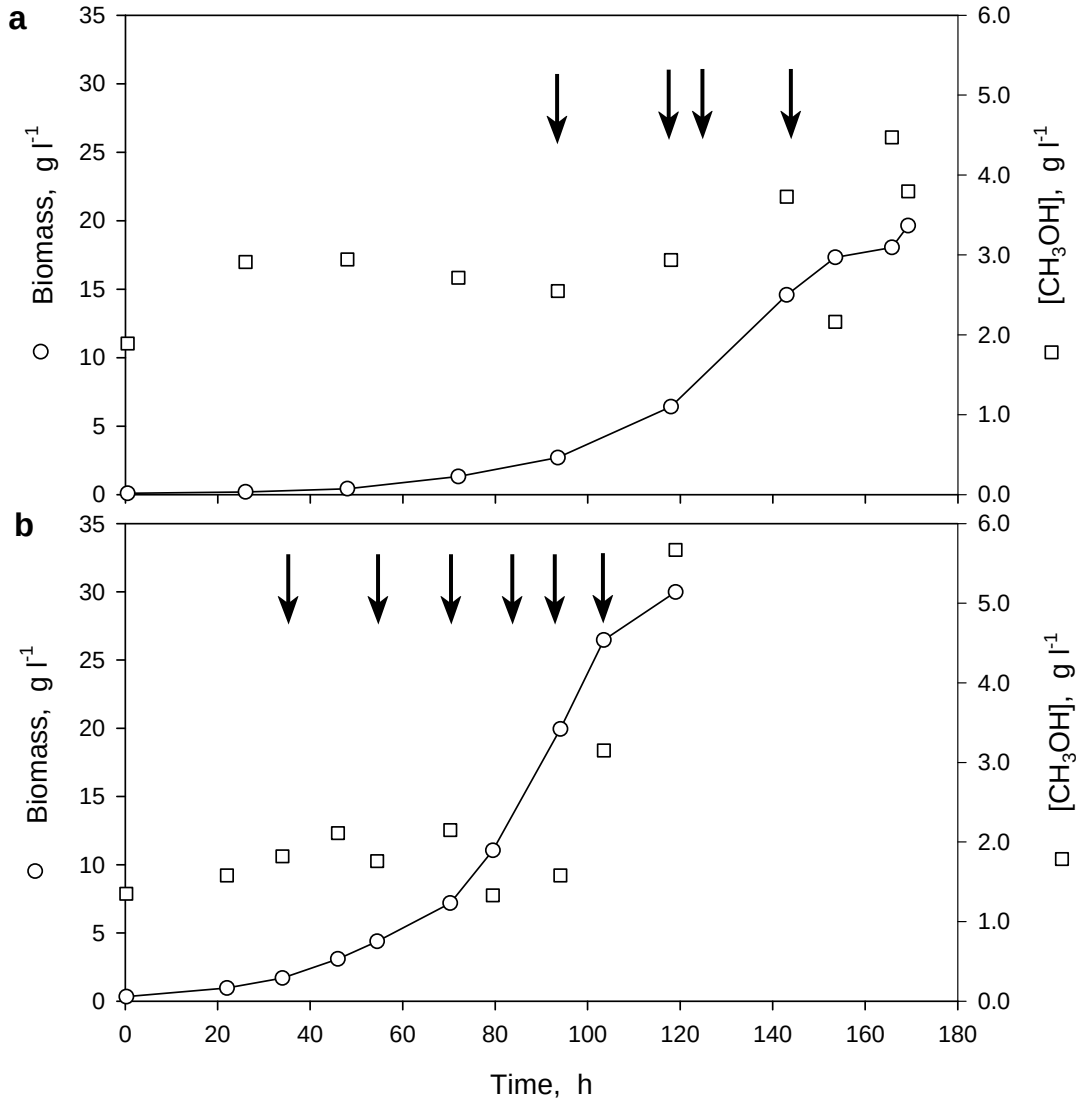

Figure 4-4 Fed-batch fermentations of *Methylosinus trichosporium* OB3b on methanol using CCP-based feeding strategy. The desired aqueous phase methanol concentration was 3 g l^{-1} (a) and 2 g l^{-1} (b). Methanol concentrations were measured directly from aqueous phase samples. At 93.5, 118, 126, and 144 h, 2 ml each of the $CaCl_2.2H_2O$, $FeSO_4.7H_2O$ and TES stock solutions were added to fermentation (a). In fermentation (b), 1 ml of each stock solution was added at 34.0 and 54.5 h and 2 ml of each stock solution was added at 70.3, 82.5, 94.1, and 103.5 h. At both 82.5 and 103.5 h, 0.34 g of additional $MgSO_4.7H_2O$ was provided.

4.5 Discussion

While a couple of studies have found that type II methanotrophs express sMMO when grown on methanol (Best and Higgins, 1981), most have concluded that they do not (Hou et al., 1979; Patel et al., 1980). In our chemostat experiments, there was no apparent difference in sMMO production whether *M. trichosporium* OB3b was grown on methane or on methanol. However, the microorganism did sometimes lose sMMO activity when stock cultures were maintained on mineral salts agar with methanol. Activity could be recovered by growing it on methane with copper limitation. The reason why sMMO activity is maintained while growing on methanol in some experiments but not others, remains to be determined. Never the less, the data from the present study proves conclusively that sMMO activity can be maintained in cultures growing on methanol.

This strain of *M. trichosporium* can tolerate exposure to as much as 40 g l^{-1} methanol, but 3 g l^{-1} was found to significantly inhibit growth. Therefore chemostat or well-controlled fed-batch culture are the most likely methods for the production of sMMO competent *M. trichosporium* from methanol. Control of fed-batch fermentations depends on supplying all of the nutrients at appropriate rates or concentrations. In terms of fermentation control, the key nutrient in this case is methanol. If the methanol level falls too low, growth slows due to limitation in carbon or energy, while, if there is too much methanol, growth will slow because of inhibition. As shown in Figure 4-5, before methanol feeding was stopped, the methanol concentration was above 3 g l^{-1} and the CO_2 production remained almost constant. Once methanol feeding was stopped at 157.5 h, the CO_2 production began to increase as methanol concentration dropped. When entirely deprived of methanol, the cells may enter a physiological state where recovery

of rapid growth is severely retarded even after addition of sufficient methanol. This occurred at the end of the fermentation depicted in Figure 4-3. We obtained a *M. trichosporium* OB3b biomass concentration of 29 g l^{-1} using a CCP-based strategy to control the methanol concentration. Much higher cell densities should be obtainable if the methanol concentration can be more rigorously controlled.

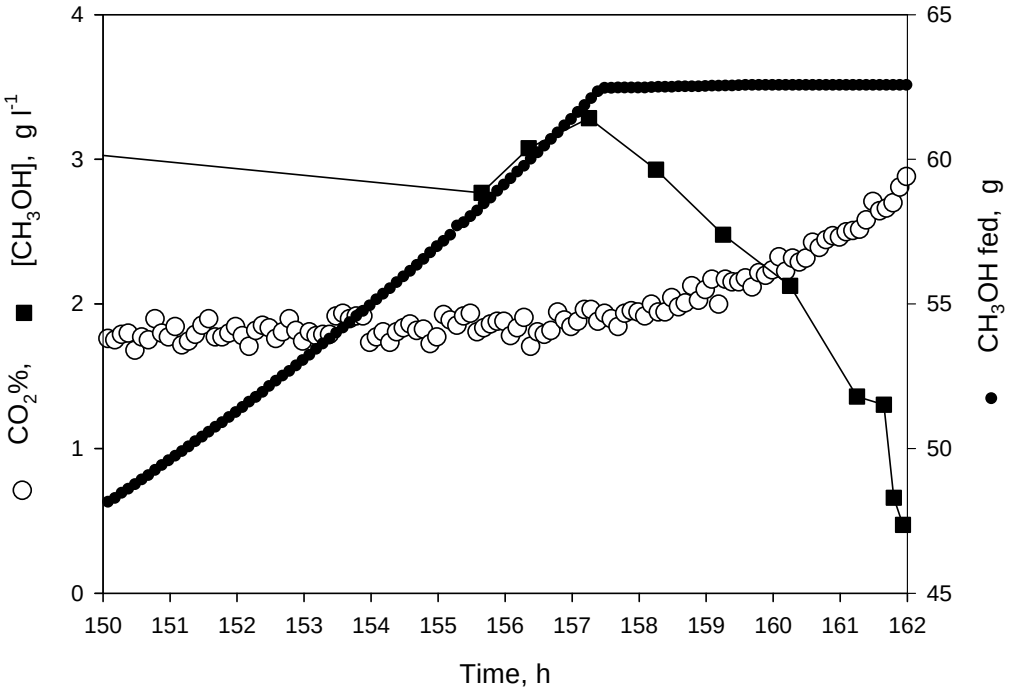

Figure 4-5 The effect of methanol concentration on carbon dioxide production during a portion of the fed-fermentation depicted in Figure 4-3.

The specific growth rate on methanol, whether in chemostat or fed-batch, was generally less than 0.1 h^{-1} even when higher feeding rates were imposed. This is similar to growth on methane. Thus, although a much higher biomass density may be obtained from growth on methanol, productivity is still limited by a low growth rate. However,

the transient μ_{CPR} reached as high as 0.3 h^{-1} at about 0.7 g l^{-1} methanol. Although the specific growth rate calculated from carbon dioxide production data may be misleading under transient conditions due to unbalanced growth, these data suggest the intriguing possibility of higher growth rates, but other factors require investigation. For example, PO_4 and other ions have been shown to inhibit methanol dehydrogenase activity (Mehta et al., 1989) and previous work (Yu et al., 2006; Chapter 3) indicated the presence of a metabolic bottleneck in this organism. However, if these barriers can be overcome, and if sufficient control of the concentration of methanol and other significant medium components during fed-batch fermentation can be maintained, it may be possible to obtain a substantial increase in specific growth rate. Even if the growth rate is not increased, substantially higher biomass concentrations can be obtained on methanol by fed-batch fermentation. This knowledge, combined with the conclusive evidence that sMMO expression can be maintained during growth on methanol should aid in the development of sMMO-based industrial and environmental processes. In future work, we intend to develop an improved process for the control of methanol during fed-batch fermentations and to monitor sMMO production throughout these fermentations. This should result in much higher biomass densities and provide greater insight regarding the effects of methlylotrophic fed-batch conditions on sMMO synthesis.

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Chapter 5

Use of Allylthiourea to Produce Soluble Methane Monooxygenase in the Presence of Copper

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Originally submitted Aug, 2008 to

Applied Microbiology and Biotechnology

5.1 Abstract

Methanotrophs expressing soluble methane monooxygenase (sMMO) may find use in a variety of industrial applications. However, sMMO expression is strongly inhibited by copper and the growth rate may be limited by the aqueous solubility of methane. In this study, addition of allylthiourea decreased intracellular copper in *Methylosinus trichosporium* OB3b, allowing sMMO production at Cu/biomass ratios normally not permitting sMMO synthesis. The presence of about 1.5 μmoles intracellular Cu g^{-1} biomass resulted in about 250 μmoles h^{-1} sMMO g^{-1} biomass whether this intracellular Cu concentration was achieved by Cu limitation or by allylthiourea addition. No loss of sMMO activity occurred when the growth substrate was switched from methane to methanol when allylthiourea had been added to growth medium containing copper. Addition of copper to the essentially copper-free medium increased the yield of dry biomass from methanol from 0.20 g g^{-1} to 0.36 g g^{-1} demonstrating that some copper is necessary for good growth. This study demonstrates a method by which sMMO can be produced by a *Methylosinus trichosporium* OB3b while growing on methanol in copper-containing media.

5.2 Introduction

Methane monooxygenase (MMO) oxidizes methane to methanol in the first step of the methane catabolic pathway in methanotrophic bacteria. There are two forms of MMO. All methanotrophs can produce membrane-bound particulate MMO (pMMO), which is believed to contain copper at its active site (Semrau et al. 1995; Zahn and DiSpirito 1996). In contrast, soluble MMO (sMMO) is a cytoplasmic enzyme found mostly in type II

methanotrophs under copper-limiting growth conditions. MMOs also differ in substrate specificity and activity (Burrows et al. 1984; Grosse et al. 1999). sMMO has a much wider substrate range than pMMO and can cometabolize the degradation of chlorinated aliphatic, aromatic and cyclic compounds (Colby et al. 1977; Oldenhuis et al. 1989; Tsien et al. 1989), including some of the most common groundwater contaminants. The potential for use of sMMO to degrade hazardous chemicals has attracted considerable interest, and it may also find use in the commercial production of certain chemicals due to its epoxidation ability (Sullivan et al. 1998). Unfortunately, trace amounts of copper ions inhibit synthesis of sMMO (Nielsen et al. 1996, 1997). Copper has also been found to inactivate the hydroxylase and reductase components of any sMMO that is synthesized (Green et al. 1985; Jahng and Wood 1996). It has been reported that sMMO is expressed only at copper concentrations less than 0.1 μM (Grosse et al. 1999), only in cells grown in the presence of less than 0.25 μM copper with a cell concentration of 0.35-0.45 g l^{-1} (Tsien et al. 1989), and that a copper concentration of 1 μM is sufficient to shift all cellular MMO activity from soluble to particulate for biomass concentrations of 0.1-0.5 g l^{-1} (Park et al. 1992). Transcription of sMMO genes may be completely repressed at copper concentrations higher than 0.86 μmol g cell (dry weight)$^{-1}$ (Jahng and Wood 1996). Since relatively low copper concentrations may have a significant effect on sMMO expression and activity, the industrial application of sMMO-producing methanotrophs has been greatly impeded. However, pMMO is known to be sensitive to a wide range of inhibitors including chelating agents, thiols, and cyanide, while sMMO is much less affected (Dalton et al., 1984; Hubley et al. 1975; Scott et al. 1981; Stanley et al. 1983). Allylthiourea is the most potent known inhibitor of pMMO (Hubley et al. 1975).

Although its effect on enzyme kinetics is well documented, methanotrophic growth and sMMO production in media containing allylthiourea has not been studied in detail. In the expectation that the copper chelating ability of allylthiourea may not only inhibit pMMO but also allow the expression and activity of sMMO, we investigated the effect of allylthiourea on sMMO activity in the type II methanotroph, *Methylosinus trichosporium* OB3b, growing on methane and on methanol in varying concentrations of copper.

5.3 Materials and methods

5.3.1 Organism and growth medium

Methylosinus trichosporium OB3b was kindly provided by Carlos Miguez of the Biotechnology Research Institute, Montreal. The organism was maintained on nitrate mineral salts (NMS) agar plates and incubated in a methane-rich atmosphere (20% CH_4 v/v). NMS consisted of $NaNO_3$, 0.85 g l^{-1}; KH_2PO_4, 0.53 g l^{-1}; $Na_2HPO_4.7H_2O$, 1.62 g l^{-1}; $MgSO_4.7H_2O$, 37 mg l^{-1}; $FeSO_4.7H_2O$, 11.2 mg l^{-1}; $CaCl_2.2H_2O$, 7 mg l^{-1}; $ZnSO_4.7H_2O$, 0.287 mg l^{-1}; $MnSO_4.7H_2O$, 0.223 mg l^{-1}; H_3BO_3, 0.062 mg l^{-1}, $NaMoO_4.2H_2O$, 0.048 mg l^{-1}; $CoCl_2.6H_2O$, 0.048 mg l^{-1}; and KI, 0.083 mg l^{-1}. The pH was adjusted to 6.88 ± 0.10 using 0.2 M H_2SO_4. Allylthiourea (98% purity) was purchased from Acros Organics, a division of Fisher Scientific, and added to the medium as specified.

5.3.2 Bioreactors and fermentation conditions

Fermentations were performed in 1.0 l of NMS in a 1.2-l fermentor (Bioflo IIc, New Brunswick) at 30°C and 700 rpm. The inoculum was 200 ml of a 96-h shake-flask culture grown on NMS medium with a 20% CH_4 (v/v) atmosphere. The bioreactor was operated in batch mode for about 72 h after inoculation and then switched to continuous

operation with an NMS feed. The dilution rate was 0.05 h^{-1} with pH controlled at 6.88 by addition of 1 N potassium hydroxide. Headspace CH_4 and CO_2 was analyzed with a Guardian infrared CH_4 analyzer (Topac Inc. Hingham, MA, USA) and a Qubit (Kingston, Ontario) infrared CO_2 analyzer. Headspace CH_4 and dissolved oxygen (DO) control, as well as data acquisition, were performed using a data acquisition board (ADR2000, Ontrak Control Systems Inc. Sudbury, ON, Canada), and a Labview™ program (National Instruments, Vaudreuil-Dorion, PQ, Canada). DO was automatically maintained above 50% of saturation. When *Methylosinus trichosporium* OB3b was grown on methanol, the methane supply was stopped and NMS medium containing 4 g l^{-1} of methanol was fed.

5.3.3 Analytical techniques

5.3.3.1 Biomass

The dry weight of biomass per unit volume of fermentation broth was determined by relating the optical density of samples at 600 nm (Spectronic Unicam UV1, Fisher Scientific, Nepean, ON, Canada) to a calibration curve.

5.3.3.2 Copper

The amount of copper in the medium, as well as any precipitated and cell-bound copper was determined as described by Fitch et al. (1993). For copper analysis, all glassware was carefully acid washed and rinsed in deionized water to remove copper contamination. After the initial methanotrophic chemostat fermentation demonstrating the effect of allylthiourea on sMMO activity, all glassware used in medium preparation was also acid washed and rinsed in deionized water. Steady-state cultures were

collected and centrifuged at 12,000 ×*g* for 4 min and the supernatant saved. The copper in this supernatant was considered to be "free copper". Pellets were resuspended in 10 mM EDTA (pH 7.0) and incubated at 200 rpm for 1 h. The suspensions were recentrifuged as before and these supernatants were also saved. The copper in this supernatant was considered to be "loosely associated with the biomass". Cell pellets were washed in water, lyophilized, and dissolved in 5 ml of 10 N HCl and then diluted in 5 ml distilled water. The copper in this fraction was considered to be "intracellular copper". Copper in all fractions was measured with an atomic adsorption spectrophotometer (Spectra AA-20 Plus, Varian) and inductively coupled plasma - optical emission spectrometer (AX-Vista, Varian).

5.3.3.3 sMMO activity

sMMO activity was determined in triplicate using a colorimetric method (Brusseau et al. 1990). Cell cultures were centrifuged and resuspended in NMS medium to a biomass concentration of 0.5 g l^{-1}. After concentrated potassium formate was added to a final concentration of 20 mM, the cell suspensions were incubated with naphthalene crystals at 200 rpm, and 30°C for 1 h. They were then centrifuged and the naphthol content was determined by adding 0.1 ml freshly prepared 1% o-dianisidine tetrazotized dye (Sigma) to 1 ml supernatant at an appropriate dilution. The development of a purple color indicated sMMO activity. 0.4 ml glacial acetic acid was added to stabilize the color (Phelps et al. 1992). The absorbance at 530 nm was measured immediately and related to a standard curve (0-48.6 μM 1-naphthol). Specific sMMO activity was calculated as the rate of 1-naphthol (μmol) formed per gram of biomass.

5.3.4 Cell morphology

Culture samples were centrifuged and washed three times with distilled water. The cell pellets were then frozen in 2.0 % para-formaldehyde and 0.5 % gluteraldehyde, embedded in JEM-EPON 812 and sectioned to a 90 nm thickness. After staining with uranyl acetate and lead citrate, cell morphology was observed with a Hitachi 7000 transmission electron microscope.

5.4 Results

5.4.1 Effect of allylthiourea on sMMO activity of *Methylosinus trichosporium* OB3b grown on methane

Without allylthiourea, *Methylosinus trichosporium* OB3b quickly lost sMMO activity whenever it was grown on NMS medium with methane as the sole carbon and energy source. Although no copper was added, the growth medium was found to contain at least 45 μg l^{-1} (0.7 μM) dissolved copper, probably from the impurities in the water and chemicals used to prepare the medium. Addition of greater than 5 moles allylthiourea $mole^{-1}$ Cu^{++} to the chemostat feed resulted in a substantial increase in the production of sMMO (Figure 5-1). Above about 20 moles allylthiourea $mole^{-1}$ Cu^{++} in the feed medium, sMMO production achieved a plateau of 720 μmol naphthol formed g^{-1} dry biomass h^{-1}.

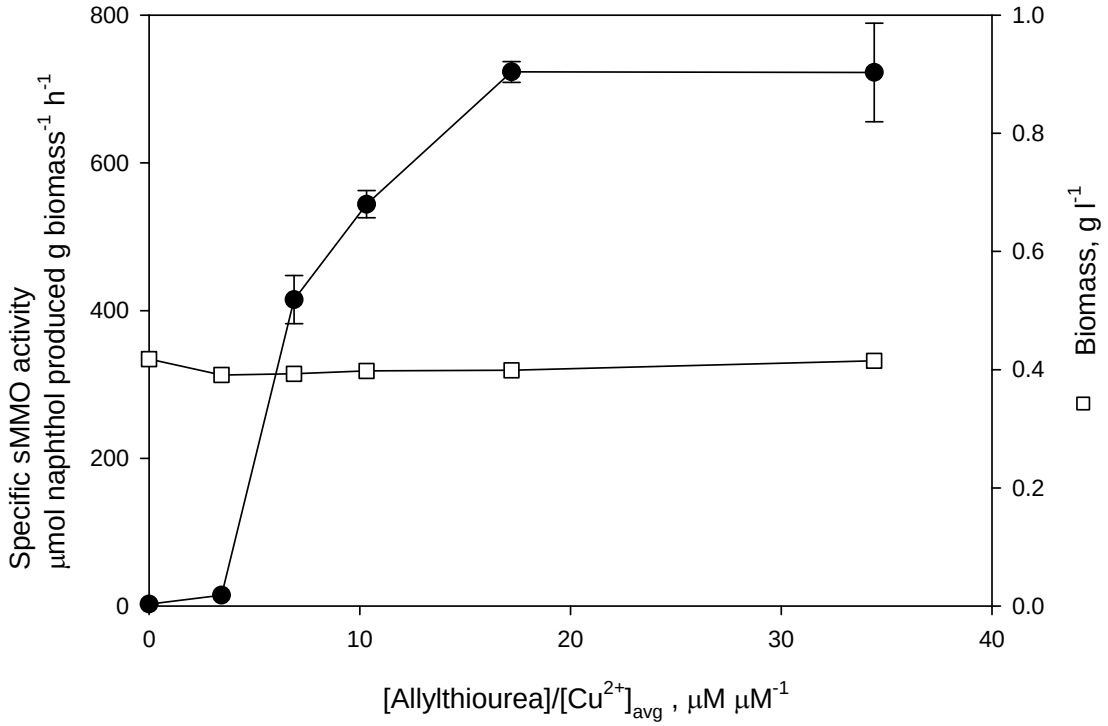

Figure 5-1 Effect of allylthiourea on specific sMMO activity of Methylosinus trichosporium OB3b grown on methane and NMS medium. Data are steady-state values obtained from a chemostat operated at a dilution rate of 0.05 h^{-1}.

5.4.2 Effect of allylthiourea on sMMO activity of *Methylosinus trichosporium* OB3b grown on methanol

M. trichosporium OB3b was grown in batch culture in NMS medium containing 15 µM of the pMMO inhibitor and the copper chelator, allylthiourea, while the headspace methane concentration was maintained at 22%. Chemostat operation began at 70 h at a dilution rate of 0.05 h^{-1} (Figure 5-2). At a steady state (judged by near constant methane uptake rate, carbon dioxide production rate, biomass concentration, and sMMO activity), a sMMO activity of 550 µmol of naphthol produced g $biomass^{-1}$ h^{-1} was

obtained. No intracellular membranes were observed, indicating the absence of pMMO (Figure 5-3a). When NMS containing 4 g l^{-1} methanol (and still with 15 μM allylthiourea) was supplied to the reactor and methane addition stopped, the specific sMMO activity remained constant at about 520 μmol naphthol formed g^{-1} biomass h^{-1}. The culture was then maintained for three weeks with medium containing methanol but no allylthiourea. The majority of sMMO activity was retained (Figure 5-2).

5.4.3 Loss of sMMO activity with copper addition to a culture growing on methanol

The chemostat experiment was continued with the same medium (methanol, without allylthiourea) but with the addition of 1 μM copper to the NMS just before the 800 h mark (Figure 5-2). This caused the sMMO activity to decrease from 490 to 400 μmol naphthol formed g^{-1} biomass h^{-1}. The added copper also caused the biomass concentration to increase dramatically, even though methanol was always maintained at a growth limiting concentration. The yield of dry biomass produced from methanol consumed almost doubled, increasing from 0.20 to 0.36 g g^{-1}. Addition of a total of 3 μM copper to the feed caused sMMO activity to drop further, to about 250 μmol naphthol formed g^{-1} biomass h^{-1} with no further increase in biomass concentration. No sMMO activity was detected 24 h after medium containing 4.5 μM copper was fed. Furthermore, although grown on methanol, addition of 4.5 μM copper resulted in production of pMMO as seen by the presence of intracellular membranes in electron micrographs (Figure 5-3b). The specific sMMO activity decreased linearly as the copper/biomass ratio increased (Figure 5-4). Slightly more than 3 μmol copper g dry $biomass^{-1}$ was sufficient to completely suppress sMMO activity. This result is very similar to the findings of previous research (Morton et al. 2000).

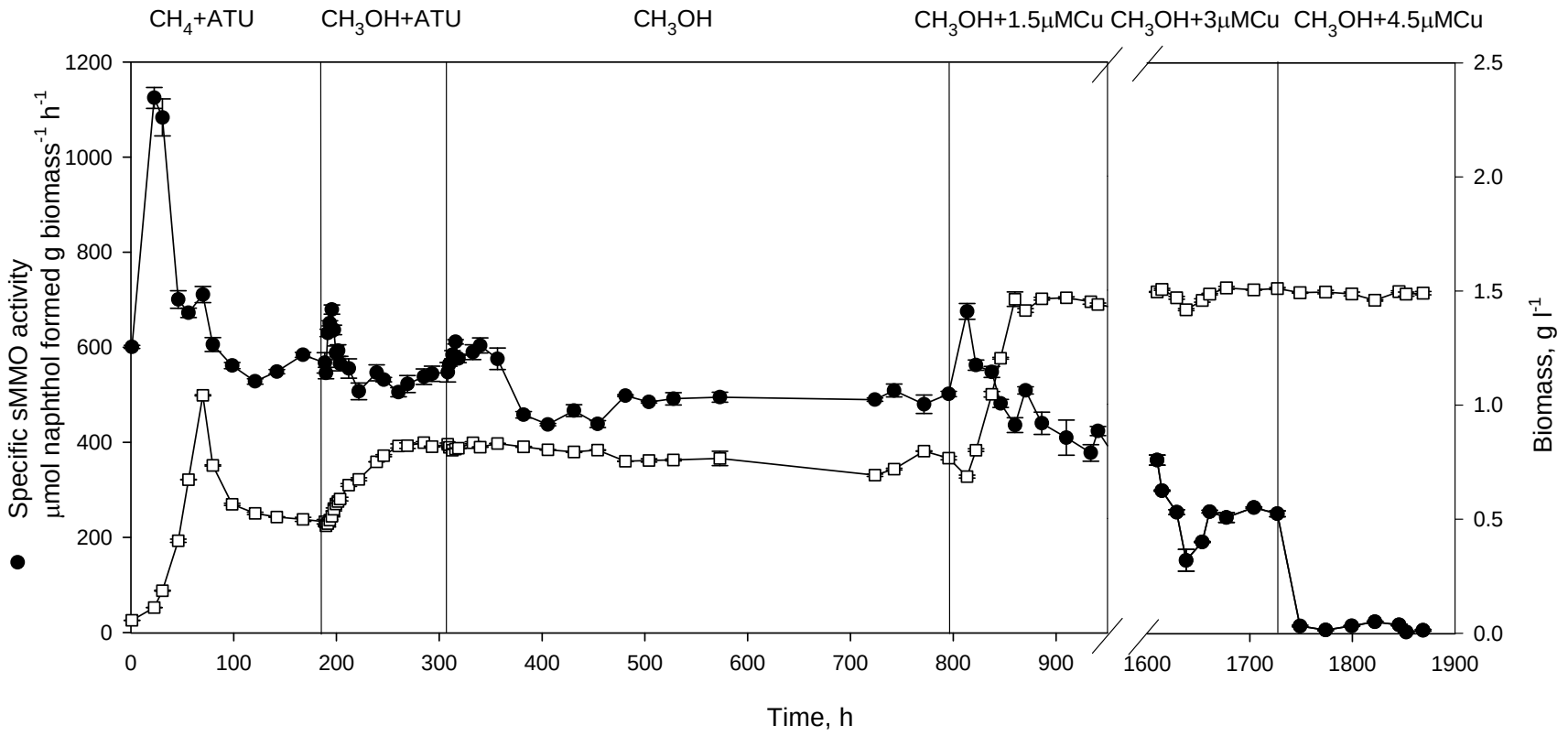

Figure 5-2 Variation of sMMO activity at different growth conditions. The culture was first grown in batch on methane with 15 μM of allylthiourea (ATU). Continuous flow of the same medium began at 70 h at a dilution rate of 0.05 h^{-1}. After steady state was achieved on methane (190 h), the methane supply was stopped and a medium containing 4 g l^{-1} methanol and 15 μM ATU was continuously fed. At 309 h, ATU was omitted from the medium. At 796, 958, and 1730 h respectively, 1.0, 3.0, and 4.5 μM copper was added to the feed.

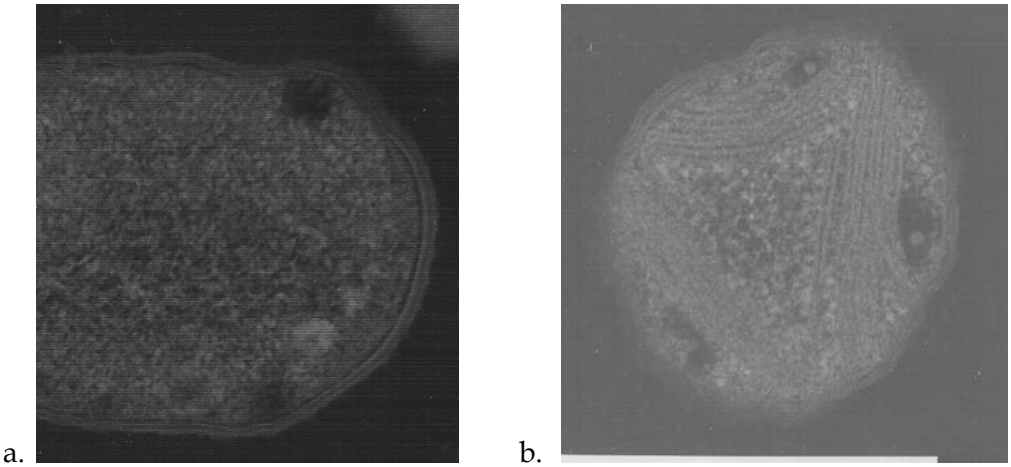

Figure 5-3 TEM of *Methylosinus trichosporium* OB3b grown under different conditions. A. Cells grown on methane with 15 μM allylthiourea (collected at 168 h in Figure 5-2). B. Cells grown on methanol with 4.5 μM additional copper in the feed (collected at 1846 h in Figure 5-2). Magnification: 70,000×.

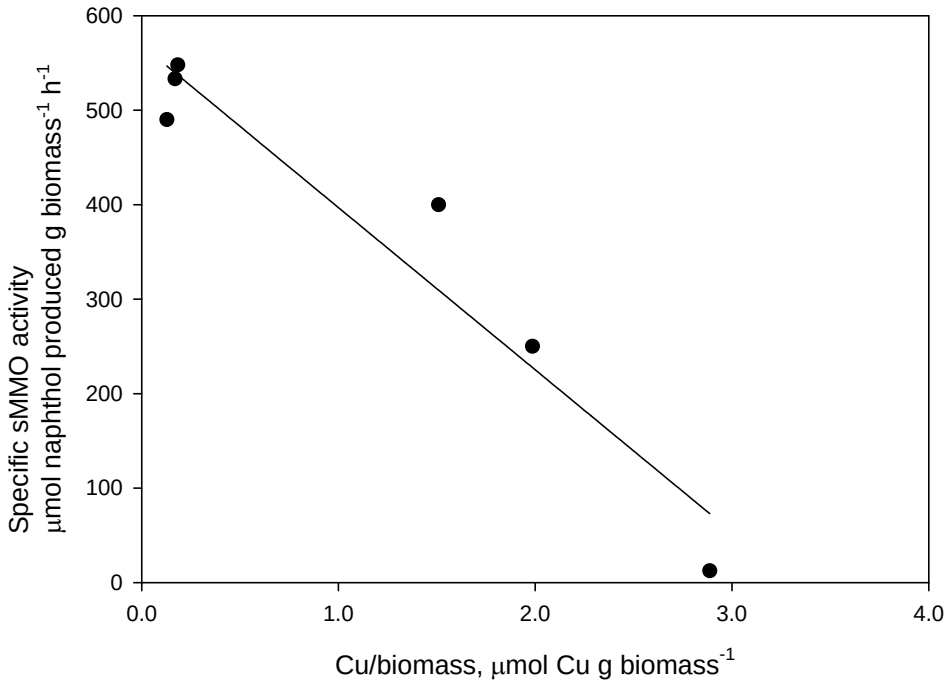

Figure 5-4 Relationship between specific sMMO activity and the total Cu/biomass ratio in the absence of allylthiourea (steady-state data from Figure 5-2).

5.4.4 Recovery of sMMO activity by addition of allylthiourea to *M. trichosporium* growing on methanol

An experiment was conducted to determine whether allylthiourea could enable production of sMMO in the presence of 4.5 μM copper. Beginning with methane but then switching to methanol as the sole source of carbon and energy, only trace amounts of sMMO were detected, if no allylthiorea was added (Figure 5-5). Addition of 15 μM allylthiourea to the NMS feed stimulated sMMO production, but at steady-state, the activity was still less than half of that obtained in the absence of copper. Although allylthiourea addition initially caused the biomass to decrease near to the level found without copper addition, biomass production gradually returned to about 1.5 g l^{-1}. Further increasing the concentration of allylthiourea in the NMS to 25 μM initially increased sMMO activity from 250 to 320 μmol naphthol formed g^{-1} biomass h^{-1} but activity then decreased rapidly and the growth rate fell to less than 0.05 h^{-1}, resulting in washout of the culture. The experiments described in section 5.4 have been repeated under similar conditions and the same trends have been obtained.

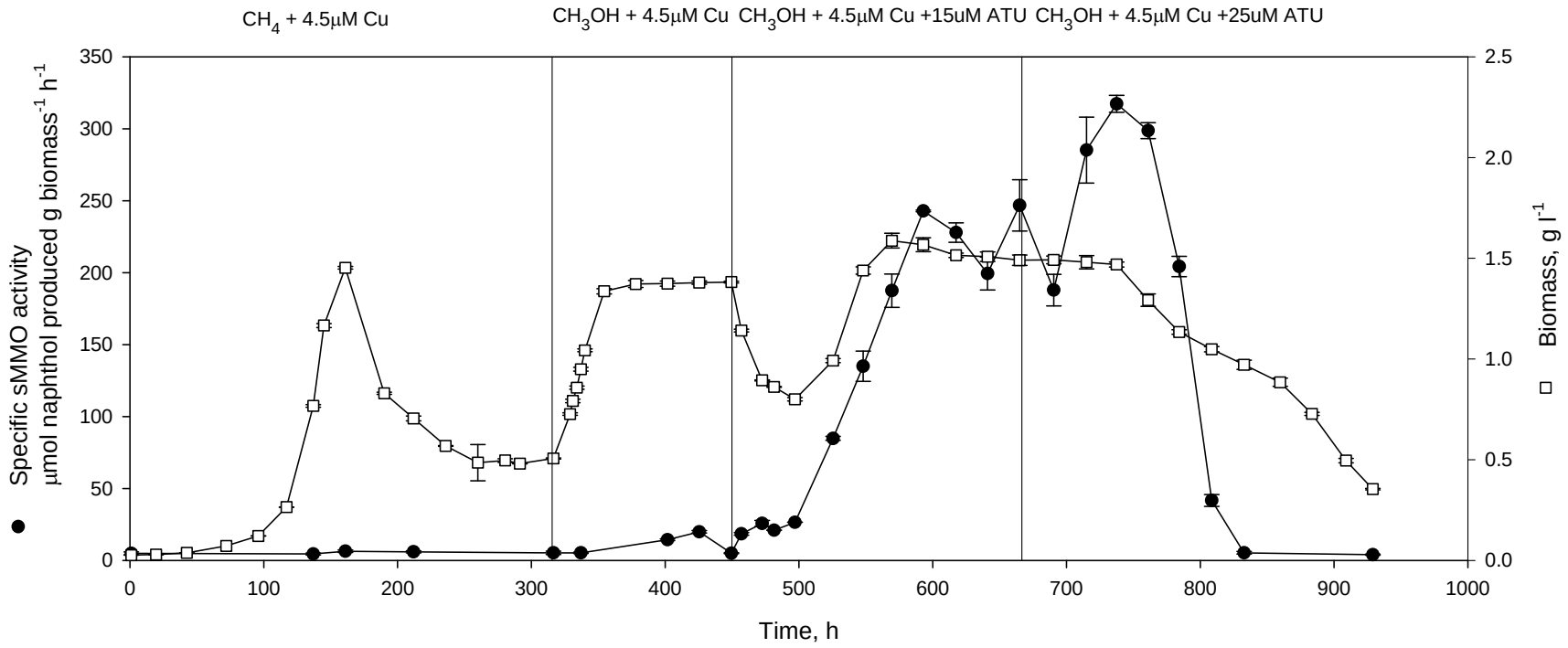

Figure 5-5 Effect of allylthiourea on recovery of sMMO activity with 4.5 μM copper in the medium. The culture was grown on methane in batch with 4.5 μM copper in the NMS medium. Continuous flow of the 4.5 μM copper-containing medium began at 161 h. Methane supply was stopped and a medium containing 4 g l^{-1} methanol and an additional 4.5 μM copper was continuously fed beginning at 317 h. Medium containing the same level of copper and methanol but with 15 μM allylthiourea was fed at 450 h. At 667 h the allylthiourea concentration in the feed was increased to 25 μM. For all the continuous flow experiments, the dilution rate was controlled at 0.05 h^{-1}.

5.4.5 Copper distribution

After the initial methanotrophic chemostat experiment (data shown in Figure 5-1), all glassware was acid washed. Thus, unless more was added, the medium after this point contained only about 0.1 μmol copper. Very little copper was in solution, although the addition of allylthiourea did increase this amount somewhat (Table 5-1). When no allylthiourea was added, intracellular copper accounted for about 80 % of the total copper. Allylthiourea addition (15 or 25 μmol) decreased intracellular copper to about 48 % (Table 5-1).

Table 5-1 Copper distribution in steady state cultures grown on methanol (from data shown in Figure 5-2 and Figure 5-5)

ATU	Cu added to influent	Free Cu	Intracellular Cu	Intracellular Cu	Intracellular Cu	Other Cu**	Total Cu	Biomass	sMMO activity
μM	μM	μM	μM	% of Total	μmoles g^{-1} biomass	μM	μM	$g\ l^{-1}$	μmol h^{-1} g biomass^{-1}
15	0	N/D	-	-	-	-	0.11	0.81	520
0	0	N/D	-	-	-	-	0.09	0.80	490
0	1.5	N/D	1.24	79	0.86	0.33	1.57	1.44	400
0	3.0	N/D	2.65	85	1.79	0.44	3.09	1.48	250
0	4.5	N/D	3.39	79	2.75	0.90	4.28	1.49	6
15	4.5	0.39	1.92	48	1.27	1.69	4.00	1.51	227
25*	4.5	0.55	1.87	47	-	1.56	3.98	<1.49	320

*Unsteady state data taken before washout.

**Insoluble or loosely associated with biomass.

N/D indicates undetectable amounts while the dashes (-) indicate concentrations that were too low or too variable to be statistically significant.

5.5 Discussion

Many studies have examined the use of sMMO for industrial applications, but in the last decade research in this area has diminished. The commercial application of sMMO has been limited by inefficient production due to mass transfer limitations of the sparingly water soluble gas, methane, as well the fact that sMMO is produced only under strict copper limitation. We have recently demonstrated conclusively that *M. trichosporium* OB3b can grow in high density culture on methanol (unpublished data). The present study now shows that chelators such as allylthiourea can be used to counteract the effects of copper concentrations commonly found in groundwater. The finding that this effect also works with cells growing on methanol greatly increases the possible commercial applications. Both particulate and soluble MMOs were produced when methanol was the sole source of carbon and energy, indicating that these enzymes do not require methane induction.

It is well known that in the absence of externally added chelators, there is a direct relationship between the amount of copper in the medium and sMMO activity. This association was again demonstrated in the present study (Figure 5-1). Addition of allylthiourea alters this relationship by reducing the amount of intracellular copper (Table 5-1). In the absence of allylthiourea, most of the copper is found inside the cell, probably due to the activity of the chelator produced by the OB3b itself (Téllez et al. 1998). Allylthiourea addition prevents entry of copper into the cells. Instead it is loosely bound to the exterior of the cell (Table 1). Although not enough data is available for accurate quantification, it is clear that there is a relationship between the amount of intracellular copper and sMMO activity. For example about 1.5 μmoles Cu g^{-1} biomass

results in about 250 μmoles h^{-1} sMMO g^{-1} biomass whether this intracellular copper concentration is achieved by copper limitation or by allylthiourea addition (Table 5-1). The dramatic effect of copper limitation and, temporarily at least, of allylthiourea addition on biomass yield, demonstrates the requirement of copper for optimal growth. For any controlled industrial process using sMMO, enough intracellular copper should be present to allow good growth while also permitting high sMMO production.

The use of copper-chelating enzyme inhibitors such as allylthiourea for practical purposes such as *in-situ* bioremediation remains challenging as they have a variety of environmental effects, some of which are obviously harmful. Allylthiourea and related compounds form chelates with other metals apart from copper and act as free radical scavengers (Hyman et al. 1990). As seen in this work, high concentrations of allylthiourea inhibit growth of *Methylosinus trichosporium* OB3b. They do not only act upon methane monooxygenases, but many other enzymes, especially copper-containing ones such as ammonia monooxygenases, and cytochromes. Removal of these chelators is necessary (via biodegradation or other means), before fermentation broth can be used for bioaugmentation or disposed into sewers. If this barrier can be overcome, pMMO inhibitors could become useful tools in the manipulation of industrial methanotrophic processes as well as academic studies. To demonstrate this concept, we are currently applying this technology to the operation of an immobilized soil bioreactor for the remediation of trichloroethylene contaminated groundwater.

5.6 References

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Chapter 6

Use of Allylthiourea and Methanol to enhance Biodegradation of Trichloroethylene in an Immobilized Soil Bioreactor in the presence of Copper

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To be submitted to Biodegradation

6.1 Abstract

A biofilm developed from a methane-enriched soil culture immobilized in a polyester fiber matrix in an immobilized soil bioreactor (ISBR) was manipulated for methanotrophic degradation of aqueous trichloroethylene (TCE). Under methanotrophic conditions without allylthiourea, no significant TCE degradation was observed indicating negligible soluble methane monooxygenase (sMMO) activity. Since even trace amounts of copper strongly inhibit sMMO expression, a copper-chelating particulate MMO inhibitor (allylthiourea) was added to selectively enrich for sMMO-producing methanotrophs in the presence of 0.47-0.71μM (0.030-0.045 ppm) Cu^{++}. Selective enrichment on methane and increased biomass production on methanol produced a culture effective at TCE degradation. In a batch experiment with an initial TCE concentration of 55.5 μM (7.3 mg l^{-1}) and 20 mM sodium formate, about 63% of TCE was removed in 8.75 h with 38% mineralization as determined by Cl^- produced. Methanotrophic activity was lost but then successfully regenerated after the immobilized culture was re-fed methane and methanol in the presence of allylthiourea. Subsequent TCE degradation in the continuous mode with only TCE and formate in the feed resulted in a maximal TCE degradation rate of 10.6 μM h^{-1} (1.40 mg l^{-1} h^{-1}) when operated at a dilution rate of 0.15 h^{-1}. This study clearly demonstrated that allylthiourea can effectively restore sMMO activity in the presence of Cu^{++}, that sMMO activity can be maintained after growth on methanol and allylthiourea with subsequent TCE degradation and, that if exhausted, this activity can be recovered. Furthermore, it was shown that this strategy can be applied to an ISBR to treat TCE-contaminated water.

6.2 Introduction

Chlorinated hydrocarbons, such as trichloroethylene (TCE), are among the most common groundwater contaminants. Although there has been extensive research regarding *in situ* treatment, current remediation practice relies mainly on the pump-and-treat approach. The most common of these processes are air stripping and activated carbon sorption where the contaminants are merely transferred from groundwater to another phase, and advanced oxidation which is expensive and relies on strong oxidizers and ultraviolet radiation to break down contaminants. Biodegradation of TCE through cometabolism is a possible alternative. Although other bacteria are able to cometabolize TCE, methanotrophs are among the most promising. Soluble methane monooxygenases (sMMOs) produced mainly by type II methanotrophs have, by far, the highest TCE degradation rates reported for any enzyme. Unfortunately, the key enzyme in methanotrophic TCE degradation, sMMO, is very sensitive to copper. Even trace amounts of copper will adversely affect sMMO expression and activity (Grosse et al., 1999; Park et al., 1992; Tsien et al., 1989). Although organisms expressing pMMO have been shown to effectively degrade TCE and may be practical for *in situ* processes (Lee et al. 2006), the specific rate of degradation is about two orders of magnitude slower than sMMO. A mutant, *Methylosinus trichosporium* OB3b PP358 was developed which can express sMMO at copper concentrations as high as 12 μM (Phelps et al., 1992). The mutant was applied to a two-stage, hollow fiber membrane bioreactor to degrade TCE in wastewater stream or in vapour (Pressman et al., 1999; Pressman et al., 2000). Unfortunately, unless a form of selective pressure is available, the use of pure cultures

requires sterilization of the medium rendering them impractical for actual bioremediation processes.

Researchers have proposed a variety of methanotrophic reactors for TCE biodegradation including suspended growth, packed-bed and expanded-bed single-stage designs. Microbial growth and TCE degradation may occur in the same reactor (Fennell et al., 1992; Speitel and Mclay, 1993; Strandberg et al., 1989). However, since both methane and contaminants are fed simultaneously, loss of TCE through volatilization and competitive inhibition of TCE degradation are inevitable. Two-stage continuous or sequencing batch processes were developed to avoid these problems. Two-stage reactor systems include a growth reactor (CSTR) and a degradation reactor (typically a plug flow reactor (PFR)) (Alvarez-Cohen and Mccarty, 1991; Chang and AlvarezCohen, 1997; Fathepure and Vogel, 1991; Mcfarland et al., 1992; Pressman et al., 1999; Sipkema et al., 1999; Tschantz et al., 1995). Cells from the CSTR are mixed with TCE-containing steam and enter the PFR. Formate is usually added to provide reducing equivalents to maintain activity. Cells in the PFR effluent are either wasted or recycled to the growth reactor. In sequencing batch reactors, biofilms are usually established on support material and microbial growth/reactivation and TCE degradation are alternated (Fitch et al., 1996; Shimomura et al., 1997; Speitel and Leonard, 1992).

The immobilized soil bioreactor (ISBR) is based on the concept of fixing soil particles containing naturally occurring microorganisms capable of degrading the target contaminants (Karamanev et al., 1998). The ISBR has been demonstrated to be extremely effective in the biodegradation of pentachlorophenol (Karamanev and Samson, 1998; Karamanev et al., 1999). In that case the soil was from a site contaminated with

pentachlorophenol. Soil particles often contain microbial consortia capable of degrading TCE (Hourbron et al., 2000; Wilson and Wilson, 1985). An ISBR containing soil from an aerobic/anaerobic interface rich in organic material was shown to be effective in methanotrophic TCE degradation (Ramsay et al. 2001), but only in the absence of copper. The ISBR provides a large surface area for slow-growing methanotrophs at low expense, and the mixed culture in the soil biofilm may help to degrade toxic intermediates from TCE degradation, thus offering protection to the methanotrophs. However, as in any bioreactor system, the amount of biomass that can be maintained is limited by the poor aqueous solubility of methane. We have recently found that allylthiourea, a pMMO inhibitor, allows significant sMMO activity in the presence of copper, and that the type II methanotroph, *Methylosinus trichosporium* OB3b, can be grown to high density on methanol while maintaining sMMO production (unpublished data). In the present study, we examined and compared the ability of a methanotrophic mixed culture (enriched soil) to degrade TCE in an ISBR in the presence of copper. The approach used was to develop the methanotrophic consortium on methane using allylthiourea to avoid copper inhibition of sMMO production. This was followed by increasing the amount of active biomass by feeding methanol. The resulting cultures were then tested for the ability to degrade TCE in both batch and continuous modes.

6.3 Material and methods

6.3.1 ISBR setup and nitrate salts medium

A 1.2 l fermentor (Bioflo II, New Brunswick Scientific) was modified as described by Yu et al. (2006) to operate as an ISBR with the on-line acquisition of Dissolve Oxygen (DO),

gas phase methane and carbon dioxide concentrations, and gas flow rates as well as the control of DO and gas phase methane levels. Gas was recirculated to provide agitation with only enough gas entering and exiting the fermentor to allow the targeted dissolved oxygen and methane levels to be maintained. During TCE degradation studies, the carbon dioxide analyzer and its desiccators were removed from the gas recirculation loop so that all reactor components in contact with the gas phase were either glass or stainless steel to minimize TCE adsorption. The nitrate mineral salts (NMS) medium consisted of (in g l^{-1}) $NaNO_3$, 0.85; KH_2PO_4, 0.53; $Na_2HPO_4.7H_2O$, 1.62; $MgSO_4.7H_2O$, 0.037; $FeSO_4.7H_2O$, 0.0112; $CaCl_2.2H_2O$, 0.007; and 1 ml/liter of trace element solution containing (in g l^{-1}) $ZnSO_4.7H_2O$, 0.287; $MnSO_4.7H_2O$, 0.223; H_3BO_3, 0.062; $NaMoO_4.2H_2O$, 0.048; $CoCl_2.6H_2O$, 0.048; and KI, 0.083. The pH was adjusted to 6.88 ± 0.10 using 0.2 M H_2SO_4.

6.3.2 Soil immobilization

Soil, collected from the aerobic–anaerobic interface of a swampy area near Kingston, Ontario, was wet sieved using a 50 mesh sieve. A measured quantity (20 to 100 g) was suspended in 500 ml distilled water and evenly dispensed onto the inert fibrous matrix (0 to 32 layers of polyester fiber furniture stuffing, Walmart) contained in the stainless steel screen (dimensions described in Chapter 3) of the ISBR. The filtrate was collected and re-applied onto the matrix five times. After gently washing off any soil particles not trapped in the matrix, the total amount of soil trapped in the matrix was determined by the difference in dry weight 24 h at 110^{o}C before and after soil immobilization.

6.3.3 Soil enrichment and batch startup of the ISBR as a methanotrophic reactor

In an enrichment step, 20 g of the above soil was incubated for at least one week in 500 ml of NMS medium in a 2 l Erlenmeyer flask at 30°C and 200 rpm on a New Brunswick gyrorotary shaker. The headspace had been purged for five minutes with 22% methane in air and sealed with a rubber stopper. The enriched soil was then added to the top of the polyester matrix in the stainless steel frame and the filtrate reapplied for a total of five times. The frame was then fixed in the center of the ISBR to which 1.2 l of NMS medium was added. Methane and air to the reactor were controlled to maintain a headspace methane concentration of 22% (v/v) and a DO above 20% of saturation. When air could not satisfy the oxygen demand, 50% oxygen or pure oxygen was used instead of air. Methane uptake rate (MUR) and carbon dioxide production rate (CPR) were monitored using an infrared CH_4 analyzer (Guardian Plus methane monitor, Topac Inc. Hingham, MA, USA) and a CO_2 analyzer (S153, Qubit Systems Inc. Kingston, Ontario, Canada), respectively. Temperature was maintained at 30°C.

6.3.4 Methanotrophic growth and TCE degradation without allylthiourea

After 71 h of operation in batch mode with 22% methane in the headspace, NMS medium was fed continuously at a dilution rate of 0.05 h^{-1} to eliminate suspended solids from the reactor. After the CPR and MUR reached steady state, the continuous feed of NMS medium and methane to the reactor were stopped (at 180 h) and 55.5 µM (7.3 mg l^{-1}) TCE and 20 mM sodium formate were added to the ISBR. A flow rate of 0.5 ml min^{-1} of air or oxygen was used to maintain the DO above 20% of saturation.

6.3.5 Methanotrophic/methylotrophic growth and TCE degradation with allylthiourea

6.3.5.1 Growth

The ISBR was prepared and operated in batch mode as described for the methanotrophic operation above except that 15 µM allylthiourea was initially present and at 145.5 h, another aliquot of 15 µM allylthiourea was added. At 162 h, methane supply to the reactor was stopped and a continuous feed of 3 g l^{-1} methanol and 15 µM allylthiourea in NMS was added at a dilution rate of 0.15 h^{-1}. At 245 h, the continuous feed was stopped and 152 µM (20 mg l^{-1}) TCE and 20 mM sodium formate were added to the ISBR. After the batch degradation of TCE at 268 h, methanotrophic activity was recovered by supplying 22% methane to the headspace in batch mode with 15 µM allylthiourea added at 270.5 h. After 12.5 h of methane addition (280.5 h), the methane supply was stopped and a continuous feed of 3 g l^{-1} methanol and 15 µM allylthiourea in NMS at a dilution rate of 0.15 h^{-1} was resumed until the CPR achieved a steady state. At 353 h, the methanol feed was replaced with 152 µM TCE and 20 mM sodium formate in NMS medium at a dilution rate of 0.15 h^{-1} with 4% methane in air at a flow rate of 1.67 ml min^{-1}.

6.3.5.2 Abiotic TCE loss

Abiotic TCE loss was determined in the ISBR loaded with unenriched soil in batch in continuous mode. The gas and liquid flow rates and temperature were the same as in the TCE degradation experiments. TCE concentrations in both the headspace and aqueous phase were measured and reported as the total TCE in the reactor.

6.3.6 Analyses

6.3.6.1 TCE and methane

ISBR headspace methane and TCE concentrations were assayed by direct injection of 100 µl of gas using a gas-tight syringe to a gas chromatograph (Hewlett Packard 5890) with flame ionization detector (FID) and a HP-5 column with helium as the carrier gas. The following temperature settings were used: isothermal oven temperature at 90°C; injector temperature at 120°C; and detector temperature at 250°C.

Aqueous phase methane and TCE concentrations were determined by gas-liquid equilibrium. A known sample volume (5-10 ml) was placed in a 12.8 ml serum bottle pre-loaded with 50 µl sodium azide (100 g l^{-1}), quickly sealed with a Mininert® valve (Supelco Canada, Mississauga, ON, Canada) and allowed to equilibrate at 200 rpm and 30°C for 1 h. A 100 µl gas phase sample from the serum bottle was then analysed by gas chromatography as described above. Quantification was achieved by relating the peak areas with those from prepared standard solutions. The detection limit is 1 mg l^{-1} for TCE and the lowest methane concentration measured was 0.15 mg l^{-1}.

6.3.6.2 Chloride

Effluent chloride concentration was measured by ion chromatography using a Dionex DX-300 Gradient Chromatographic System equipped with Ionpac AS4A guard and analytical columns connected to a conductivity detector. Samples were filtered through 0.45 µm polycarbonate filters before injection. The mobile phase was 1.7 mM sodium bicarbonate and 1.8 mM sodium carbonate at a flow rate of 1 ml min^{-1}. The calibration curve of standard chloride solutions was in the range of 0 to 100 mg l^{-1}.

6.4 Results

6.4.1 Soil immobilization

The operation of the ISBR may be affected by the amount of soil that can be loaded onto the support matrix. Therefore, the amount of soil that could be immobilized and the effect of the number of layers of the porous support on soil immobilization were studied. The amount of soil that could be trapped in the fibrous matrix of the support material was directly related to the number of layers of material (Figure 6-1). Increasing the amount of fiber in a fixed geometry effectively reduces the pore spaces in this matrix, allowing smaller particles to be trapped. Increasing the amount of soil loaded eventually saturates the available space as is evident from the decreased percentage of soil immobilized at the highest soil loading (100 g). In all subsequent experiments, 20 g of soil suspended in 500 ml NMS medium and enriched in 22% methane was loaded onto 24 layers of geotextile by the same method.

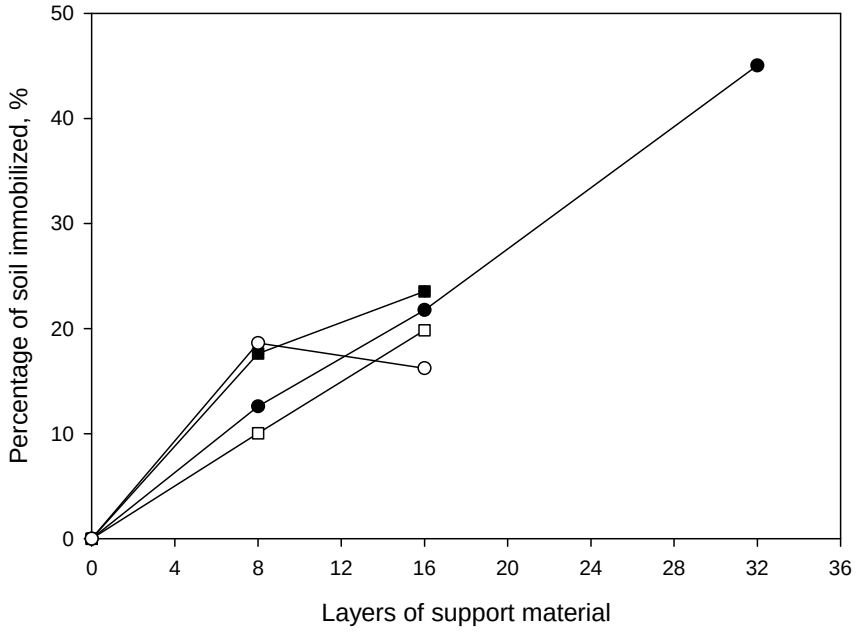

Figure 6-1 Amount of soil immobilized when 20 (●), 40 (□), 70 (■) or 100 (○) g of soil was loaded onto the ISBR geotextile

6.4.2 Methanotrophic growth and TCE degradation in ISBR without allylthiourea

Supplying methane and air to the methane-enriched soil immobilized on the polyester matrix resulted in a steady increase in methanotrophic activity (Figure 6-2a). Continuous flow of NMS medium began at 71 h resulting in a decrease in suspended soil particles and planktonic bacteria and decreased MUR and CPR which stabilized at about 1900 and 1200 μmol h^{-1} respectively. The aqueous phase Cu^{2+} concentration was 0.47-0.71 μM (0.030-0.045 mg l^{-1}). At 180 h, continuous flow was stopped and 55.5 μM (7.3 mg l^{-1}) TCE was added. There was no significant TCE degradation compared to an abiotic control performed under identical conditions at a different time (b).

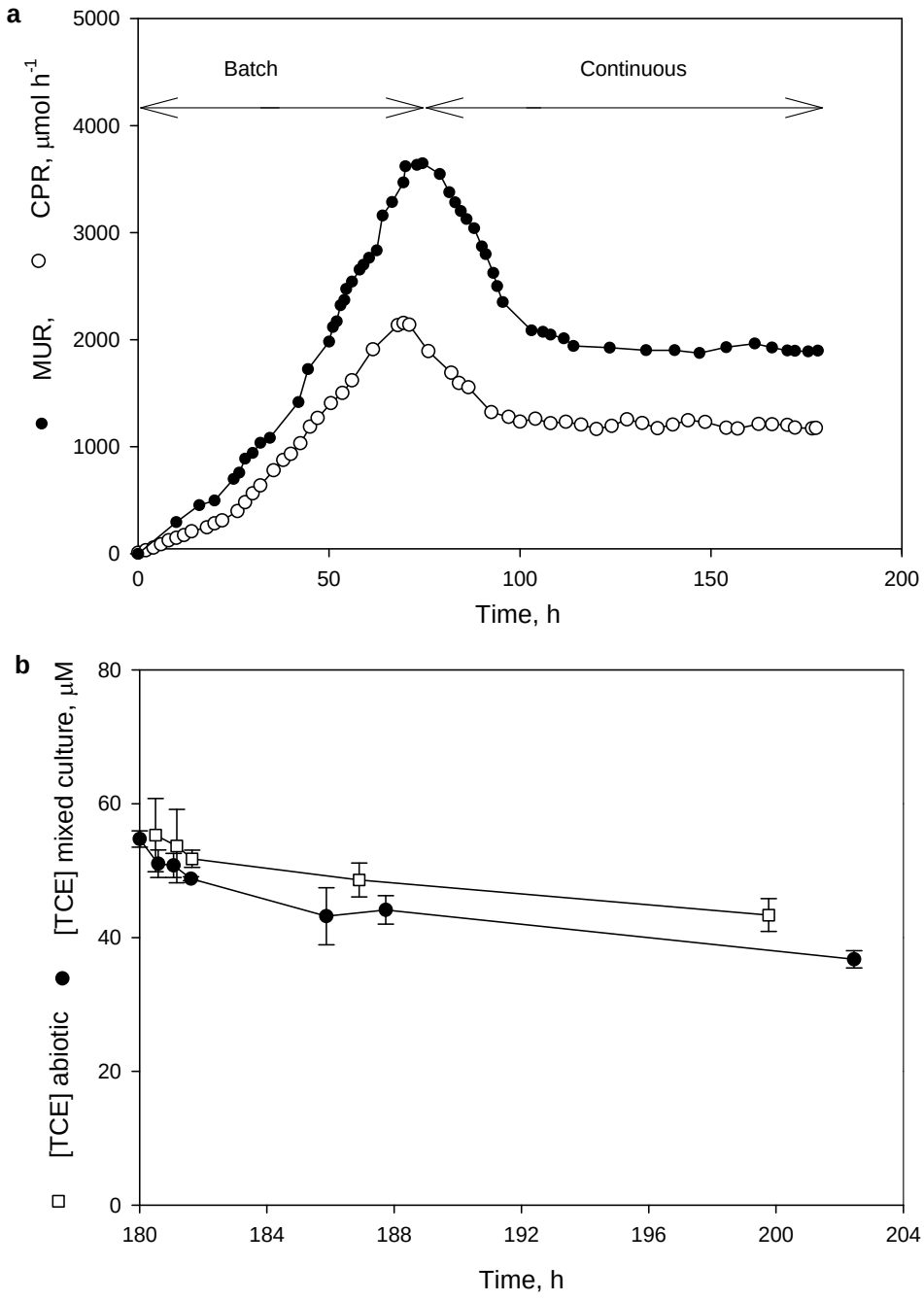

Figure 6-2 (a) Methanotrophic growth of methane-enriched soil culture immobilized in ISBR in NMS medium (no allylthiourea) and 22% methane. At 71 h, continuous flow of NMS medium without allylthiourea began at a dilution rate of 0.05 h^{-1} with 22% methane. At 180 h, arrow indicates that the continuous flow of medium and methane flow were stopped and 55.5 µM TCE and 20 mM sodium formate added to the reactor. (b) Batch TCE degradation at 30°C and an air flow rate of 0.5 ml min^{-1}.

6.4.3 Effect of allylthiourea on methanotrophic/methylotrophic growth and TCE degradation in an ISBR

Since there was no methanotrophic TCE degradation in the presence of 0.47-0.71 μM (0.030-0.045 mg l^{-1}) Cu^{2+}, the experiment was conducted again under identical conditions but with 15 μM of allylthiourea in the NMS medium. The presence of allylthiourea produced a long lag period of about 90 h followed by a rapid increase in the methanotrophic activity (Figure 6-3a). Addition of another 15 μM allylthiourea at 145.5 h caused an immediate decrease in CPR from 1525 to 292 μmol h^{-1} indicating that about 70% of the earlier methanotrophic activity was probably due to pMMO. A subsequent increase in the CPR without a lag phase to 1449 μmol h^{-1} in 16.5 h indicated an increase in the sMMO-producers.

The methane supply was stopped at 162 h and a continuous feed NMS medium containing methanol and allylthiourea was begun. The subsequent transient decrease in CPR was again probably due to the removal of planktonic bacteria and suspended soil particles with perhaps some adaptation to methanol. The CPR stabilized at 1300 μmol h^{-1} (Figure 6-3a).

At 245 h, the continuous feed was stopped and 152 μM (20.0 mg l^{-1}) TCE and 20 mM sodium formate were added to the ISBR. This time the TCE decreased quite rapidly compared to the abiotic control. TCE disappearance was almost linear in the first 5.75 h with about 68% TCE degradation after 8.5 h (Figure 6-3b). About 60% of the decrease in TCE was due to complete mineralization as 140 μM (4.98 mg l^{-1}) Cl^- was produced.

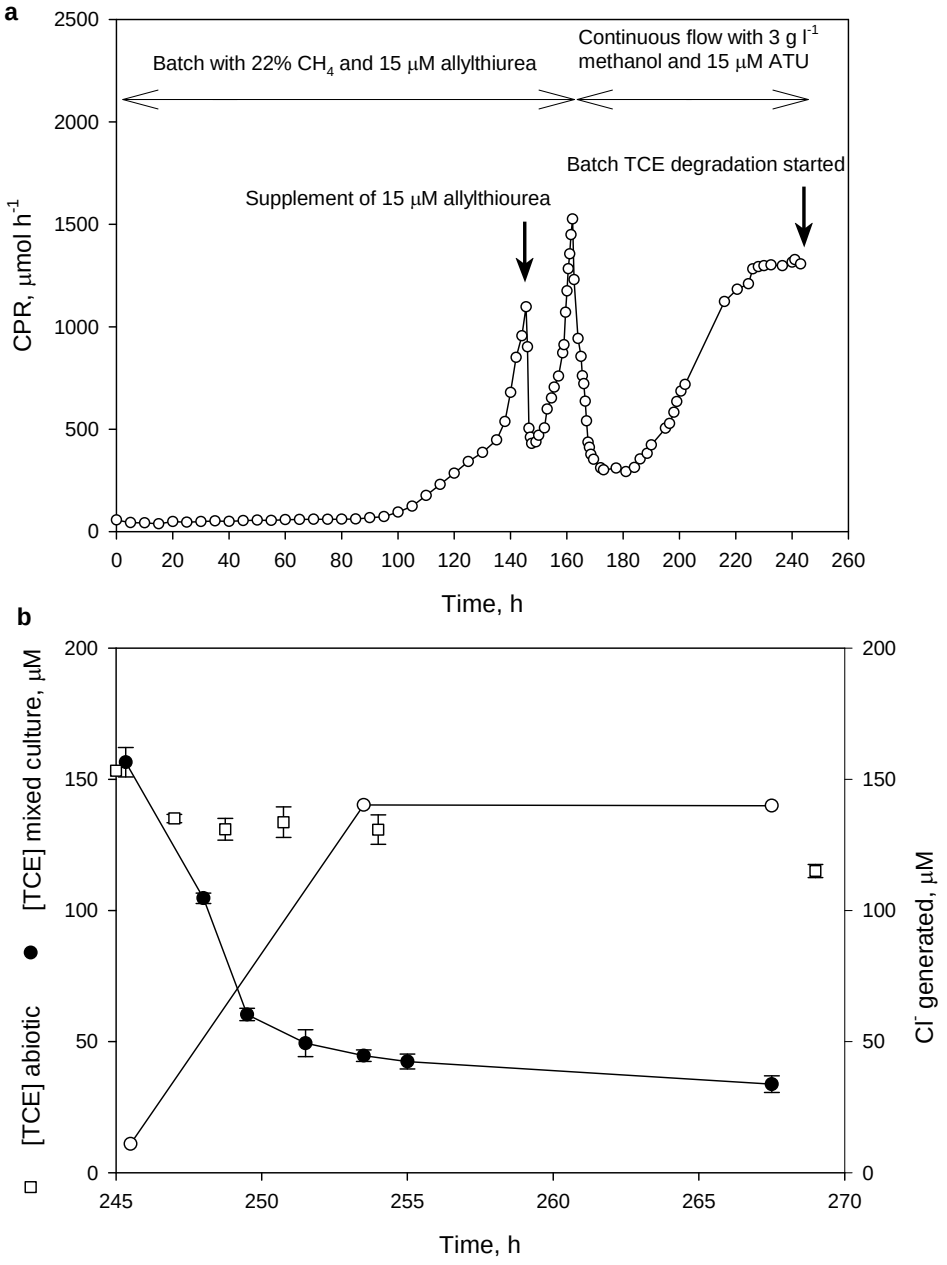

Figure 6-3 (a) Methanotrophic growth of methane-enriched soil culture immobilized in ISBR in NMS medium with 15 μM allylthiourea and 22% methane. At 145.5 h, another 15 μM allylthiourea was added. At 162 h, the methane supply was stopped and NMS medium containing methanol and 15 μM allylthiourea was fed continuously at a dilution rate of 0.15 h^{-1}. At 245 h, the arrow indicates that continuous flow of medium was stopped and 152 μM TCE and 20 mM sodium formate were added to the reactor.(b) Batch TCE degradation in ISBR after selective enrichment of sMMO-producing organisms using allylthiourea.

6.4.4 Recovery of methanotrophic/methylotrophic activity

After the batch TCE degradation in Figure 6-3b, the carbon dioxide analyzer was reconnected to the gas recirculation line. The CPR of the immobilized culture had decreased significantly from 1300 μmol h^{-1} before TCE addition to only 108 μmol h^{-1} at 268 h, clearly indicating a decrease in methanotrophic/methylotrophic activity. To regenerate methanotrophic activity, the aqueous phase in the ISBR was replaced with fresh NMS medium containing 15 μM allylthiourea and 22% methane in air was resupplied to the headspace in batch mode for 12.5 h (Figure 6-4a). Methanotrophic activity increased rapidly until 15 μM allylthiourea was added at 270.5 h (2.5 h after methane was re-supplied) (Figure 6-4a). The CPR stopped decreasing at 280.5 h (12.5 h after resupplying methane). At this time, the methane supply was stopped and a continuous flow of methanol and allylthiourea in NMS medium began. The CPR only recovered to about 1000 μmol h^{-1}, about 77% of the level that had been obtained before the TCE batch degradation experiment. Sampling with enrichment shake-flasks and agar plates containing NMS with allylthiourea as the sole source of carbon and energy produced no growth, indicating that no allylthiourea biodegradation was taking place.

6.4.5 Continuous TCE degradation

At 340 h, the methanol feed was replaced with one containing 152 μM TCE and 20 mM sodium formate in NMS medium at the same dilution rate of 0.15 h^{-1} with small amount of methane (4%) in the gas phase. There was 55-60% TCE removal by the enriched and adapted biofilm after 24-48 h (i.e. after 4 to 8 volume changes) (Figure 6-4b). Although about 55% TCE degradation was maintained for 24 h, TCE degradation gradually declined with the reactor concentration increasing to 109 μM (14.36 mg l^{-1}) at 505 h, most

likely reflecting a loss of methanotrophic activity. The maximum TCE degradation rate was 10.6 μM h^{-1} (1.40 mg l^{-1} h^{-1}).

After switching to a continuous TCE feed, the net chloride produced increased to 175 μM (6.20 mg l^{-1}) at 70.5 h and slowly dropped to 71 μM (2.53 mg l^{-1}) at 166 h of continuous feed started (Figure 6-4b) indicating a TCE mineralization of 39 and 15.5%, respectively. The influent NMS medium had a measured chloride concentration of 95 μM (3.38 mg l^{-1}).

6.5 Discussion

This work demonstrates that a methanotrophic bioreactor can be applied to TCE degradation in the presence of 0.47-0.71 μM (0.030-0.045 ppm) Cu^{++}, which is possible in groundwater (Meranger et al., 1979). It was clearly shown that by adding allylthiourea during an enrichment or regeneration phase, TCE degradation capacity can be generated. Since the sole source of carbon were methane and methanol, and since there was no significant activity without allylthiourea, this activity must be due to sMMO.

Allylthiourea acts on many copper-containing enzymes and thus is toxic to a variety of organisms. Even, as in this work, if it was employed only during batch enrichment or regeneration processes, there would be some release into the environment, albeit at low concentration. We found no evidence that allylthiourea is biodegradable. Its replacement with a biodegradable copper-chelator would be desirable.

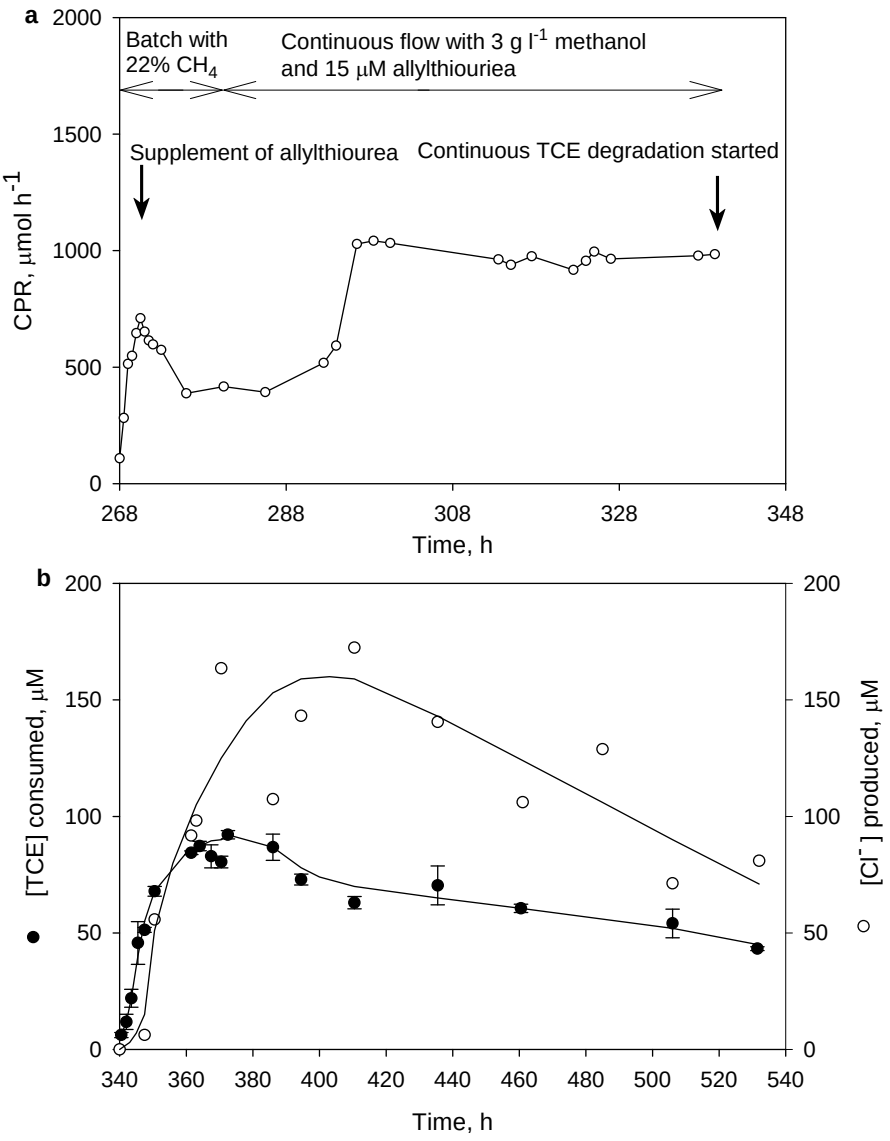

Figure 6-4 (a) Recovery of methanotrophic activity. At the end of TCE degradation in Figure 6-3b at 268 h, methane (22%) was resupplied for batch growth of methanotrophs for 12.5 h. At 270.5 h, 15 μM of allylthiourea was added. At 280.5 h, (i.e. after 12.5 h of methane resupply), the methane flow was stopped and a continuous flow of NMS medium containing 3 g l^{-1} methanol and 15 μM of allylthiourea began at a dilution rate of 0.15 h^{-1}. At 340 h, the NMS feed was replaced with NMS containing 152 μM TCE and 20 mM sodium formate at the time indicated by arrow. (b) TCE consumed and Cl^- produced in continuous TCE degradation study in the ISBR after recovery of methanotrophic activity at 30°C with NMS medium containing 152 μM (20 mg l^{-1}) TCE and 20 mM sodium formate at a dilution rate of 0.15 h^{-1} and an air flow rate of 1.67 ml min^{-1}.

Operation of an ISBR would likely be far less expensive than currently employed techniques. Never-the-less it may not be capable of reducing the concentrations of TCE (or other contaminants) to desirable levels. As seen in the batch degradation experiments, the degradation rate decreases greatly at low TCE concentration. Thus it would most likely be used as a pretreatment step to reduce the amount of TCE that must be degraded by more expensive processes. Operation of an ISBR as a sequencing batch reactor should be an effective approach as the initial high TCE concentration would result in a more rapid degradation rate. After TCE concentration reached the desired level, the aqueous phase would be released for further treatment and the methanotrophic activity regenerated. Biological processes are also temperature sensitive. Operation of methanotrophic bioreactors at groundwater temperatures should be investigated.

While use of allylthiourea produced clear results, use of methanol to increase activity did not seem to be an improvement on use of methane alone. Although, in theory, methanol should allow for higher density biomass and penetrate more easily into biofilms, other factors may limit biomass production. The CPR data indicated that steady states were achieved even though methanol in the medium should have resulted in further biomass production. A study of mass transfer of nutrients and contaminants such as TCE through the ISBR biofilm will aid in understanding the operation of this bioreactor. Never-the-less, it appears that with the aid of allylthiourea or a similar copper chelator, the methanotrophic ISBR may be suitable for practical application in processes using sMMO for the remediation of copper containing effluents.

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Chapter 7

Conclusions and Recommendations

7.1 Conclusions

- Dissolved methane concentration can be accurately measured by establishing relationship between k_La for methane and oxygen, and gas-phase methane analysis.
- High density cultivation of methanotrophs on methane is not feasible due to the low solubility of methane in water.
- High-cell density cultivation of methanotrophs can be achieved by using methanol as the growth substrate.
- *Methylosinus trichosporium* OB 3b and probably other methanotrophs (as shown by ISBR experiments) can retain their ability to produce sMMO during growth on methanol.
- Allylthiourea (ATU) and probably other copper chelators can be used to allow sMMO in the presence of copper by lowering the intracellular copper concentration.
- Allylthiourea concentrations above 25 μM inhibit methanotrophic cell growth probably due to copper limitation.
- Methanotrophic cultures producing sMMO can be enriched from mixed culture inocula such as soil by using methane as the sole source of carbon and energy and allylthiourea to reduce the intracellular copper concentration.

- A methanotrophic immobilized soil bioreactor can be used to mineralize TCE and its TCE degradation capacity can be regenerated by judicial use of ATU, methane and methanol.

7.2 Summary of scientific and engineering contributions

7.2.1 On-line estimation of dissolved methane concentration

To study the kinetics of both TCE degradation and methanotrophic growth, a rapid and accurate analysis of dissolved methane concentration was needed. A continuous on-line dissolved methane analysis was developed using a standard infrared methane gas analyzer and a dissolved oxygen (DO) probe (Chapter 3). This method provides a reliable, simple approach to estimate dissolved methane concentration on-line with an easily-available DO probe and a methane gas analyzer. This is the first method to estimate dissolved methane concentration on-line. It will help to investigate and understand the kinetics of cometabolic methanotrophic TCE degradation. This method can also be applied to other fermentation processes to measure volatile components for which probes are not available.

In developing this method, the k_La for methane and on-line estimation of methane uptake rate were determined. Our results showed that methane mass transfer rate in the bioreactors was quite low because of the poor water solubility of methane and that growth on methane as measured by the methane uptake rate was severely limited by the methane transfer rate. A more water soluble growth substrate would alleviate the mass transfer limitation.

7.2.2 High-density cell cultivation of methanotrophs

Methanol is miscible with water and there should be no mass transfer limitation. Since it is inhibitory at high concentrations to *M. trichosporium* OB3b, fed-batch cultivation was developed to achieve high-density biomass production of *M. trichosporium* OB3b (Chapter 4). Although an exponential feeding strategy based on predetermined μ achieved a final biomass of 7g l^{-1}, methanotrophic growth did not follow the pre-set μ exactly, so that starvation and over-feeding occurred.

A modified feeding strategy was successfully developed where methanol was continuously fed based on the methanol consumption rate estimated from the cumulative CO_2 production and yield of CO_2 from methanol. This feeding strategy resulted in a better biomass production and methanol control where 19 and 29 g l^{-1} of biomass were obtained by maintaining methanol concentration around 3 and 2 g l^{-1} respectively. This is the first study which has applied an automated feeding strategy to achieve high-cell density production of methanotrophs. 29 g l^{-1} is the highest biomass density that has been reported. This fed-batch feeding strategy based on carbon dioxide production is very easy to control, manipulate, and scale up. It is not only good for the cultivation of methanotrophs on methanol, but may also be beneficial to other fermentations using toxic or inhibitory substrates.

Many studies have reported that methanotrophs do not express sMMO when grown on methanol. However, our studies clearly indicated that *M.trichosporium* OB3b retained sMMO activity when grown on methanol. Further we have proved the possibility to grow methanotrophs to high density on methanol. These findings significantly enhance the practicality of methanotrophic bacteria to TCE degradation.

7.2.3 Effect of allylthiourea on pMMO inhibition and sMMO recovery

Unfortunately, *M. trichosporium* OB3b completely lost the ability to express sMMO at a Cu/biomass ratio of 3 (Chapter 5). A pMMO inhibitor, allylthiourea, was supplemented to the medium to compel the methanotrophs to synthesize sMMO even in the presence of Cu^{2+} at the amount that has been shown to fully inhibit sMMO expression. Under the same conditions in chemostat culture but in the presence of 15 μM allylthiourea, *M. trichosporium* OB3b retained half of the sMMO activity normally found without Cu^{2+}. With 25 μM ATU, a more complicated phenomenon was observed. The sMMO activity increased briefly followed by washout of the biomass.

Copper distribution analysis indicated that the addition of allylthiourea decreased intracellular copper content. Without allylthiourea, most of the Cu was found to be intracellular. Addition of allylthiourea decreased Cu bioavailability and hence helps to relieve inhibitory effect of Cu on sMMO. However our study also demonstrated Cu is required for optimal growth of methanotrophs since addition of Cu to the medium enhances the cell yield. To realize an efficient and practical methanotrophic process involving sMMO, the intracellular Cu content should be well controlled to allow for both high sMMO expression and good growth.

Despite of the high activity of sMMO towards TCE, the commercialization of methanotrophs to TCE degradation has been impeded due to poor growth on methane and the fact that sMMO is strongly inhibited by Cu. This is the first study which uses a pMMO inhibitor to maintain reasonable sMMO activity in the presence of Cu^{2+}. Allylthiourea maintained sMMO activity in the presence of Cu with methane or methanol as substrate. Our previous study (Chapter 4) also indicated that when the

growth substrate was switched from CH_4 to CH_3OH, the majority of sMMO was retained and that high density growth of methanotrophs can be obtained from methanol. These findings significantly increase the possibility of commercial applications such as on-site production of sMMO-expressing biomass for bioaugmentation or other purposes.

7.2.4 Methanotrophic TCE degradation in an ISBR

The practicality of the above achievement on high density biomass production of a methanotroph on methanol (Chapter 4) and relieving Cu^{2+} inhibition on sMMO activity using allylthiourea (Chapter 5) were applied to a bench-scale ISBR to obtain maximal degradation of TCE (Chapter 6). The ISBR was operated in a sequencing mode to enhance the efficiency of methanotrophic cometabolism of TCE by separating cell growth and sMMO production/recovery from TCE degradation. In the growth stage, methane and methanol were introduced with allylthiourea for selective enrichment of type II methanotrophs. In the degradation stage, once deterioration in sMMO activity and methanotrophic degradation of TCE was observed, the ISBR was shifted to growth stage during which period methanol or methane feed was resumed for biomass regeneration and sMMO recovery.

The capacity of the ISBR to degrade TCE was examined in both batch and continuous operation. In a batch TCE degradation experiment with 153.3 μM of initial TCE, about 67% of TCE was removed in 8 h. The maximal TCE degradation rate of 10.73 $\mu mol\ l^{-1}\ h^{-1}$ was obtained in a continuous TCE degradation at a dilution rate of 0.15 h^{-1}. Disappearance of TCE was accompanied by an increase in chloride content indicating the mineralization of TCE. The ISBR presented little or no activity towards TCE without

applying the strategy based on high-cell density cultivation and addition of allylthiourea.

The effectiveness of methanotrophic TCE degradation was demonstrated in the ISBR. The study provides an effective methanotrophic TCE degradation process including methanotrophic growth/regeneration, enzyme activity recovery, and sequential TCE degradation. The sequencing operation of ISBR allows for continuous treatment of TCE-contaminated water which is more preferable for on-site application if two or more reactors are operated in series. The efficiency of ISBR in methanotrophic TCE degradation demonstrated in our study will greatly enhance the commercialization of methanotrophic systems.

7.3 Recommendations for future work

TCE degradation by *M. trichosporium* OB3b is a cometabolic process and requires a substrate (such as methane) to induce the necessary oxygenases. The substrate can provide carbon and energy for growth and its concentration should be fed in high enough quantity to compensate for energy loss during TCE cometabolism. Since methane also competes with TCE for the enzyme active site, its concentration should be kept low to minimize competitive inhibition of TCE degradation. The kinetics of methanotrophic growth, TCE degradation, competition between the growth substrate and TCE, and enzyme activity needs to be thoroughly studied and understood. The balance between growth substrate and co-substrate is prerequisite for efficient TCE degradation.

Although 29 g l^{-1} of biomass density was been achieved (Chapter 4), it is desirable to obtain even higher biomass production with sMMO activity. High-density biomass production is not only good for TCE degradation in bioreactors in a pump-and-treat operation, but also beneficial to in situ bioremediation. The knowledge from the fed-batch fermentations for high-density biomass production, including nutrient requirements and substrate feeding strategies, can be used to stimulate indigenous bacteria *in situ*. Bioaugmentation by reactor-grown bacteria may further improve the efficiency of *in situ* bioremediation.

This study (Chapter 5) indicated that addition of the pMMO inhibitor, allylthiourea, will help to maintain sMMO activity in the presence of Cu. However, the mechanism by which allylthiourea inhibits pMMO is not clear. Is it simply that allylthiourea chelates free Cu and hence decreases Cu bioavailability or does allylthiourea interfere with Cu assimilation in methanotrophic bacteria? Further study on the effect of allylthiourea on pMMO activity and sMMO recovery and elucidating the mechanism should be done. This information can be used to further stabilize sMMO activity. It is also worthwhile to investigate whether other pMMO inhibitors can achieve better recovery of sMMO activity in the presence of Cu.

Temperature is a key factor affecting methanotrophic activity and hence the efficiency of *in situ* bioremediation. So far most methanotrophic studies have been done at mesophilic or room temperatures, while groundwater temperature is much lower (~10^{o}C). In general, microbial growth and enzyme activity decrease as the temperature decreases so that results obtained at mesophilic temperatures may not represent on-site application. Therefore to make the methanotrophic treatment system more practical, it

is important to screen for psychrophilic or psychotrophic methanotrophic bacteria with high sMMO activity.

TCE contaminated groundwater often contains a variety of other compounds such as PCE. PCE is not degraded by methanotrophs and is also an inhibitor of MMO. Reductive dechlorination is relatively efficient for heavily chlorinated CAHs, such as PCE, and the partially dechlorinated products, for instance VC, are more susceptible to aerobic degradation. Thus the combination of anaerobic reduction followed by aerobic cometabolism has the potential to completely degrade highly chlorinated compounds. Although this combination has been previously shown, it has not been evaluated in an ISBR.

An acidophilic methanotroph, *Methylocella silvestris*, was isolated from an acidic forest cambisol and expressed sMMO constitutively in the presence of copper but no pMMO was detected (Dunfield et al., 2003). This bacterium was also reported to have greater tolerance to methanol. This bacterium and other methanotrophic species able to express sMMO constitutively in the presence of copper are good candidates to be evaluated in an ISBR as the process may be simpler to control and operate.

7.4 References

Dunfield PF, Khmelenina VN, Suzina NE, Trotsenko YA, Dedysh SN. 2003. *Methylocella silvestris* sp nov., a novel methanotroph isolated from an acidic forest cambisol. Int J Syst Evol Microbiol 53:1231-1239.