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REVIEW

Biodegradation of Chlorinated Hydrocarbons by Facultative Methanotrophs

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Bioremediation is one of the most effective ways to treat and dispose of chlorinated hydrocarbons and methanotrophs are potentially useful to do so. Recent studies found that facultative methanotrophs can use compounds containing C-C bond as sources of carbon and energy, thus overcoming the limitation that obligate methanotrophsone uses only C1 compounds for this process. This is a unique metabolic approach that is becoming increasingly attractive in the field of contaminant biodegradation. Here, we summarized the discovery process of facultative methanotrophs the bioremediation of chlorinated hydrocarbons by obligate and facultative methanotrophs. This process involves the degradation of various chlorinated hydrocarbons by diverse strains, including pure cultures and mixed cultures. We also compare the activity expression and catalytic properties of different types of methane monooxygenases in various substrates. We furthermore summarize the kinetic characteristics of the degradation of chlorinated hydrocarbons using the model strain *Methylosinus trichosporium* OB3b and outline the degradation and potential of chlorinated hydrocarbons by facultative methanotrophs. Lastly, we discuss current problems and future research directions for degradation of chlorinated hydrocarbons by methanotrophs.

Keywords: Facultative methanotrophs, Methane monooxygenase, Bioremediation, Chlorinated hydrocarbons, Kinetics.

INTRODUCTION

Aerobic methanotrophs are widely distributed in the environment, including in wetlands, bogs, agricultural, forest and urban soils, rice paddies, groundwater, landfill cover soils, among many other locations[1]. These cells play a critical role in the global carbon cycle by utilizing methane as a source of carbon and energy and it is estimated to consume atmospheric 30 Tg (Tg = 1012 g) methane each year[2]. Besides methane, in contrast, facultative methanotrophs could utilize other mutil-carbon compounds as a source of carbon and energy[3]. Although reports about facultative methanotrophs was observed in the 1970s, the fact that facultative methanotrophs was exist in the world has been widely recognized up to this day. The isolated facultative methanotrophs included *Methylocella*, *Methylocystis* and *Methylocapsa* which were α-proteobacteria[4-6]. Many reports have recently been published of methanotrophs that also able to utilize multi-carbon compounds as sole growth substrates. Hence, it appears that facultative methanotrophs may be more common than originally thought.

Volatile chlorinated hydrocarbons (VCH), such as trichloroethylene (TCE) and chloroform (CF), seriously polluted ecological environment and threat to human health because of unreasonable application in industry and human life[7,8]. Facultative methanotrophs have unique physiological and substrate selectivity characteristic that can degrade various chlorinated hydrocarbons[9-12] and methane monooxygenase (MMO) play a key role in degradation process[13]. Most of methanotrophs possess particulate methane monooxygenase (pMMO), but few can express both soluble methane monooxygenase and particulate methane monooxygenase[14]. Difference from scope's substrate, affinity for substrate and sensitivity to inhibitors of the two enzymes led to diversity of catalytic degradation of oxidizing chlorinated hydrocarbons. Substantial studies on degradation of chlori-nated hydrocarbons were performed, including biodegradation of different chlorinated hydrocarbons[15,16], the reaction kinetics and mechanism[17,18] and application on biodegradation of chlorinated hydrocarbon *etc.*[19,20].

In this paper, we summarize progress and discovery process of facultative methanotrophs and the bioremediation of chlorinated hydrocarbons by obligate and facultative methanotrophs systematacially. It involves the degradation various chlorinated hydrocarbons by diverse strains, including pure cultures and mixed cultures. We also compared the activity expression and catalytic properties of different types of methane monooxygenases in various substrates. We

furthermore summarized the kinetic characteristics of the degradation of chlorinated hydrocarbons using the model strain *Methylosinus trichosporium* OB3b and outlined the degradation and potential of chlorinated hydrocarbons by facultative methanotrophs. Lastly, we discussed current problems and future research directions for degradation of chlorinated hydrocarbons by methanotrophs.

Discovery process of facultative methanotrophs: As shown in Table-1, as early as 1970, Whittenbury found that methanotrophs, *Methylocystis sporium*, *Methylocystis methanica* and *Methylocystis albus* show enhanced growth on methane when malate, acetate, or succinate was also present in the culture medium, such findings suggested that facultative methanotrophs may exist[21,22]. After 10 several years, the reports involved facultative methantrophs appeared. However some strains lost the ability to oxidize methane when grown repeatedly on culture[21,22], or some strains appear to not be still extent, making it impossible to repeat these experiments, even one of these isolates was later found by members of the same laboratory to actually consist of a stable syntrophic consortium of two methylotrophs[23,24]. The lack of extant strains and evidence of stable mixed cultures initially originally described as pure methanotrophic strains all cast serious doubts on the possibility of facultative methanotrophy. As a result, research in this area was severely limited for the next 20 years. Efforts to identify novel methanotrophs didn't significantly regain momentum until discover the *Methylocella palustris*[5] which was a new genus and species within Alphaproteobacteria in 1998.

After that, *Methylocella silvestris* and *Methylocella tundrae*[6,25,26] were isolated subsequently, then cultures of these strains were unequivocally shown to be pure through a suite of rigorous assays, including: (1) phase-contrast analyses of thousands of cells grown with either acetate or methane; (2)

TABLE-1
DISCOVERY PROCESS OF FACULTATIVE METHANOTROPHS

Strains	Discoverer	Discovery time	Discovery area	Metabolic characteristics	Conclusions
Gram-negative, strictly aerobic methane-utilizing bacteria	Whittenbury *et al.*[3]	In 1970	-	A wide variety of methanotrophs, *sporium, methanica* and *albus* were enhanced growth on methane when malate, acetate, or succinate was also present in the culture medium	Facultative methanotrophs may exist
Methylobacterium organophilum	Patt *et al.*[14]	In 1974-1976	Freshwater lake sediments and water	These could utilize a wide range of multicarbon compounds as growth substrates, including many organic acids and sugars. This strain, however, lost the ability to oxidize methane when grown repeatedly on glucose, and other workers subsequently did not succeed in growing the strain on methane [21-22]	They were not facultative methanotrophs
Methylobacterium ethanolicum strain R6	Patel *et al.*[2, 30-31]	In 1978	An oil refinery in the Northeastern United States	These strains were able to grow solely on glucose, but not with other sugars such as fructose, galactose, or sucrose	None of these strains is still extant, making it impossible to repeat these experiments
Methylobacterium ethanolicum *Methylobacterium hypolimneticum*	Lynch *et al.*[14, 32]	In 1980	Freshwater lake sediments	They able to utilize not only methane, but also casamino acids, nutrient agar, and a variety of organic acids and sugars for carbon and energy	It was later found to consist of two methylotrophs *Methylocys* and *Xanthobacter*.[24]
					It was not reported later
Methylomonas sp. strain 761M/761H	Zhao and Hanson[2]	In 1984	A rice paddy in South China	761M only could grow on methane, but 761H could not grow on glucose as the sole carbon source, and glucose, as well as acetate and malate, were reported to enhance its growth on methane	Didn't find the same strain, and finally could not determine
Methylocella palustris	Dedysh *et al.*[5]	In 1998	*Sphagnum* peat bogs	It was the first characterized acidophilic methanotroph which brought significantly regained momentum to identify novel methanotrophs	It was a new genus and species within *Alphaproteobacteria*
Methylocella silvestris BL2	Dunfield *et al.*[6]	In 2003	Cambisol under a beech-dominated forest stand near Marburg, Germany	These methanotrophs, however, were later shown to be facultative as they could utilize not only C1 compounds for growth, but also acetate, pyruvate, succinate, malate, and ethanol	It was the first time that was shown to be facultative methanotroph in 2005[26,33]
Methylocella tundrae	Dedysh *et al.*[23]	In 2004	Acidic Sphagnum tundra peatlands		
Methylocapsa aurea	Dunfield *et al.*[27]	In 2010	A soil sample collected in March 2003 from under a small ephemeral brook in a forest near Marburg, Germany	It was identified that could utilize acetate as the sole growth substrate. However, M. *aurea* only expresses pMMO	*Methylocapsa* was a novel facultative methanotrophs[14,27]
Methylocystis strain H2s/ *heyeri* H2	Belova *et al.*[4]	In 2011	A sample collected in July 2001 from 10 cm below the surface of *Sphagnum* peat	It possesses both forms of methane monooxygenase (particulate and soluble MMO) and a well-developed system of intracytoplasmic membranes (ICM), it able to grown on acetate absence of methane	*Methylocystis* was also a novel facultative methanotrophs.
Methylocystis, strain SB2	Im *et al.*[4]	In 2011	A spring bog in southeast Michigan	It was able to utilize methane, ethanol, or acetate as growth, and can only express pMMO substrates	
Methylocystis strain H2s^T, *Methylocystis* strain S284	Belova *et al.*[28]	In 2012	An acidic (pH 4.3) Sphagnum peat-bog lake (Teufelssee, Germany) and an acidic (pH 3.8) peat bog (European North Russia)	They possess both a soluble and a particulate methane monooxygenase, The preferred growth substrates are methane and methanol. In the absence of C1 substrates, however, these methanotrophs are capable of slow growth on acetate	

sequence analyses of 50 16S rRNA gene clones from both acetate- and methane-grown cultures; and (3) whole-cell hybridizations of thousands of cells with probes specific for *Methylocella*. In no case was any evidence of contamination found. Furthermore, real-time PCR assays showed increases of the mmoX gene (encoding for the large hydroxylase subunit of the soluble methane monooxygenase) that very closely corresponded with direct microscopic cell counts.

As shown in Fig. 1[26,27], whole-cell hybridization in a culture of *Methylocella silvestris* grown on acetate as the sole carbon and energy source was performed. Upper panel, phase contrast; middle panel, hybridization with the *Methylocella* genus-specific probe Mcell-1445; lower panel, hybridization with the *Methylocella silvestris* species-specific probe Mcells-1024. Together, these tests are strong evidence for culture purity. These study definitely confirmed that facultative methanotrophs was isolated.

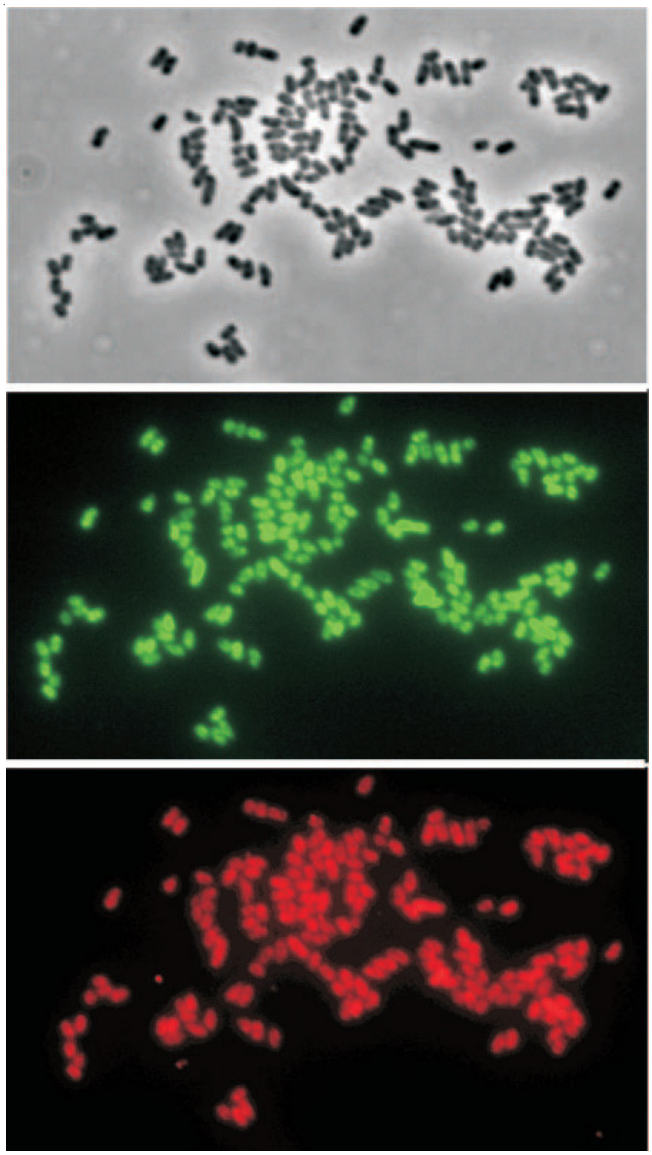

Fig. 1. Whole-cell hybridization in a culture of *Methylocella silvestris* grown on acetate as the sole carbon and energy source

Shortly thereafter, *Methylocapsa* 27 and *Methylocystis* 4 were also isolated and suggested to be facultative methanotrophs. In contrast to *M. silvestris*, the newly acidophilic methanotroph, *Methylocapsa aurea* only expresses pMMO and have well-developed ICM system, *M. aurea* grew best on methane, with a maximum OD_{600} = 1.2, μ_{max} = 0.018 h^{-1}.

It is worth mentioning that *Methylocapsa aurea* could utilize acetate and ethanol as the sole growth substrate and expressed pMMO during assimilating these compounds[14,27]. Belova *et al.*[4] found that a acidophilic methanotrophs, which belong to genus *Methylocystis* (family *Methylocystaceae*) with both soluble methane monooxygenase and pMMO, could grow on either methane or acetate. It was found to not only utilize methane and methanol for growth, but also the maximum OD_{410} nm of 0.8-1.0 and 0.25-0.30, respectively and growth rate was 0.06 h^{-1} and 0.006 h^{-1}. These data indicated that methane was the growth substrate for *Methylocystis* H2s, however, strain H2s was not found to grow significantly on any other organic acid or sugar as well as other acidophilic methanotrophs. Thereafter Belova *et al.*[4] screened validly described *Methylocystis* species *Methylocystis heyeri* H2 for facultative methanotrophic growth, which also grew significantly on acetate. Belova *et al.*[28] isolated two facultatively methanotrophic representatives of the genus *Methylocystis*, strains $H2s^T$ and S284 again and they possess both a soluble and a particulate methane monooxygenase, the preferred growth substrates are methane and methanol. In addition, an mesophilic *Methylocystis* species, *Methylocystis* SB2 which was able to utilize methane, ethanol, or acetate as growth and can only express pMMO substrates was reported by Im and Shemrau[25]. Growth was highest on methane followed by ethanol and acetate (maximum OD_{600} nm of 0.83, 0.45 and 0.26, respectively).

According to the reported facultative methanotrophs, we found that *Methylocella silvestris* (BL2) capable of growth at pH values between 4.5 and 7 (with an optimum at pH 5.5)[6], *Methylocella* tundrae capable of growth between pH 4.2 and 7.5 (optimum 5.5-6.0) 23, *Methylocapsa aurea* KYG^T grew at pH5.2-7.2[27] and *Methylocystis* H2s was mesophilic with optimum pH 6.0-6.5. The optimum pH of *Methylocystis* heyeri H2[27] and *Methylocystis* SB2[29] were 5.8-6.2 and 6.8, respectively. *Methylocystis* strain H2sT and S284 grew at pH5.2-7.2 6.0-6.5[28]. These results indicated that facultative methanotrophs were well-grown in acid condition and the optimum pH was 5.5-6.5, as shown in Fig. 2.

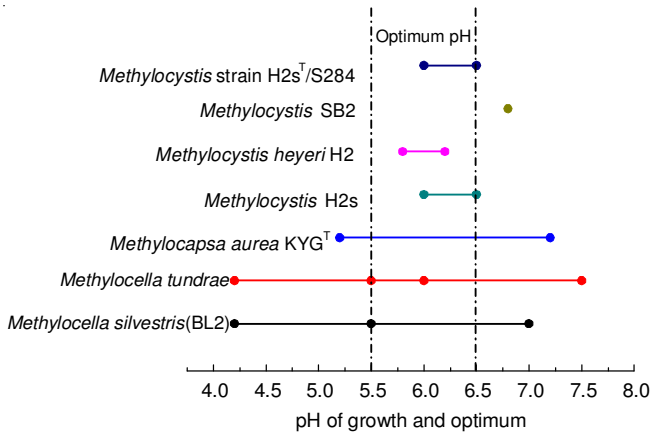

Fig. 2. growth and optimum pH of facultative methanotrophs

Degradation kinetics of chlorinated hydrocarbon by methanotrophs: Pettipher *et al.*[34] found that the methanotrophs-riched free soil column which was exposed to a mixture of natural gas in air was able to degrade trichloroethylene, which broke the traditional view that methanetrophs can only use methane and promoted the discovery of facultative methanotrophs. In the ensuing decades, researchers explored the degradation of chlorinated hydrocarbon by different methanotrophs which included mixed bacteria and pure bacteria and the pollutant was not limited to one chlorinated hydrocarbon. Fogel *et al.*[35] studied the degradation of chlorinated hydrocarbon including trichloroethylene by mixed methanotrophs isolated from sediment sample. Biodegradation experiments shown that approximately half of the radioactive carbon had been converted to $^{14}CO_2$ and bacterial biomass in 1986. In 1988, degradation of trichloroethylene was also studied using Strain 46-1, a type I methanotrophic bacterium, degraded trichloroethylene if grown on methane or ethanol by Little *et al.*[36]. Later, out of seven chlorinated hydrocarbon degradation by mixed culture and two pure bacterials *Methylomonas methanica* NCIB11130 and *M. trichosporium* OB3b were compared by Deboosere *et al.*[37]. From the consortium, they were all capable of degradation of *trans*-1,2-dichloroethylene (*t*-1,2-DCE) and no significant difference, which indicated that methanotrophs were dominant in the process. Researchers studied the degradation of trichloroethylene by methanotroph *Methylocystis* sp. strain M successively[38-40], distinct difference that the result in degradation of trichloroethylene by mixed culture MU-81 and *Methylocystis* sp. strain M and then an non-autotrophic bacteria strain DA4 was isolated. Thus, it indicated that this microorganism played an important role in this process. So it came to light that various non-methanotrophs existed in mixed culture and the function of them was different.

Catalytic properties of methane monooxygenase on degradation of chlorinated hydrocarbon

Exploration on degradation of chlorinated hydrocarbon by methane monooxygenase: For a long time, how chlorinated hydrocarbon was degraded by methane monooxygenase is still an intractable problem perplexing researchers. Some studies indicate that NADH-dependent monooxygenase is derepressed when cells are grown under copper stress and pMMO is expressed by all methanotrophs whereas soluble methane monooxygenase is expressed only by type II methanotrophs under copper-limited conditions[15,41], but soluble methane monooxygenase has a wider specificity than the particulate form. A substantial amount of work concerning methanotrophic co-metabolic transformations has been carried out using the soluble form of methane monooxygenase from the obligate methanotroph *Methylosinus trichosporium* OB3b. Soluble methane monooxygenase can degrade trichloroethylene (TCE) at a rate that is at least one order of magnitude faster than that obtained with other mixed and pure cultures, which suggested it is more suitable for bioremediation[42].

Transformation yields for the aerobic co-metabolic degradation of five kinds of chlorinated ethenes were determined using a methanotrophic mixed culture expressing pMMO. The yields for t-DCE and VC were 20 times greater than the yields reported by others for cells expressing soluble methane monooxygenase. Transformation yields for c-DCE, trichloroethylene and 1,1-DCE were similar to or less than those for cultures expressing soluble methane monooxygenase, which indicated that affinity is difference for different substrates although with the same enzyme. Degradation of trichloroethylene by the methanotrophic acterium *Methylosinus trichosporium* OB3b was studied using cells grown in continuous culture by Oldenhuis[13]. Results shown that trichloroethylene was degraded when grown under copper limitation and when the SMMO was derepressed which demonstrated that trichloroethylene degradation was a strictly co-metabolic process, requiring the presence of a co-substrate, preferably formate and oxygen. Furthermore, the study of Tsien *et al.*[43] shown that when expressing of soluble methane monooxygenase was inhibited, the specific rates of methane and methanol oxidation did not change during growth, while the ability of trichloroethylene oxidation increased with the appearance of soluble methane monooxygenase. These conclusions also proved that trichloroethylene was catalyzed and degraded by soluble methane monooxygenase indirectly and the conclusions determined by Western blot (immunoblot) analysis with antibodies prepared against the purified enzyme. Oxidizing of chlorinated, fluorinated and brominated alkenes by the soluble purified from *M. trichosporium* OB3b was studied by Fox *et al.*[44], the oxidation rates for the chloroalkenes were observed to be comparable to that for methane, the natural substrate and up to 7000-fold higher than those reported for other well-defined biological systems. Jahng and Wood[45] also found that this enzyme oxidizes the most frequently detected pollutant, trichloroethylene (TCE), at least 50 times faster than other enzymes.

Factors on degradation of chlorinated hydrocarbon by soluble methane monooxygenase and its diversity: Soluble methane monooxygenase can degrade many halogenated aliphatic compounds that are found in contaminated soil and groundwater. However, slow growth of the strain, strong competition between trichloroethylene and methane for soluble methane monooxygenase and repression of the smmo locus by low concentrations of copper ions limit the use of this bacterium. To overcome these obstacles, the 5.5-kb smmo locus of *M. trichosporium* OB3b was cloned into a wide-host-range vector (to form pSMMO20) by Jahng and Wood[45] and this plasmid was electroporated into five *Pseudomonas strains*. The best trichloroethylene degradation results were obtained with *Pseudomonas putida* F1/pSMMO20. The maximum trichloroethylene degradation rate obtained with the recombinant strain was lower than that of *M. trichosporium* OB3b but greater than other trichloroethylene-degrading recombinants and most well-studied *pseudomonads*. In addition, this recombinant strain mineralizes chloroform (a specific substrate for soluble methane monooxygenase), grows much faster than *M. trichosporium* OB3b and degrades trichloroethylene without competitive inhibition from the growth substrate. Results of Fox and Jahng's studies shown that application of soluble methane monooxygenase on project is feasible and genetic engineering generating some novel breakthrough was promising future. Lee *et al.*[46] found that despite such an apparent growth advantage, pMMO-expressing cells degraded less of these substrates at these concentrations than soluble methane monooxygenase-expressing cells during active growth. And

at concentrations of VC, t-DCE and trichloroethylene greater than 10 µmol/L each, methanotrophs expressing pMMO have a competitive advantage over cells expressing soluble methane monooxygenase due to higher growth rates, if the concentrations were increased to 100 µmol/L, however, not only did pMMO-expressing cells grow faster, they degraded more of these pollutants and did so in a shorter amount of time. From these findings, it appears that the relative rates of growth substrate and pollutant degradation are important factors in determining which form of methane monooxygenase should be considered for pollutant degradation.

Methylosinus trichosporium OB3b mutants having constitutive expression of soluble methane monooxygenase was isolated by Strand *et al.*[47] and one mutant (PP358) exhibited a trichloroethylene degradation rate which was almost twice as high as that of the wild-type strain grown under optimal conditions (without copper). Koh *et al.*[48] isolated an methanotroph *Methylomonas methanica* 68-1 and strain 68-1 was found to oxidize naphthalene and trichloroethylene *via* a soluble methane monooxygenase (sMMO) and thus becomes the first type I methanotroph known to be able to produce this enzyme. The substrate affinity of 68-1 soluble methane monooxygenase to trichloroethylene however, was comparatively lower than that of the soluble methane monooxy-genase of OB3b, which had affinities of 40 ± 3 and 126 ± 8 µmol/L, respectively, OB3b showed that the soluble methane monooxygenase genes of 68-1 have little genetic homology to those of OB3b. This result may indicate the evolutionary diversification of the soluble methane monooxygenase.

Soluble methane monooxygenase catalyzes a range of oxidation reactions, including the hydroxylation of alkanes, epoxidation of alkenes and the oxidation of ethers, halogenated methanes and cyclic and aromatic compounds[49-51]. However, many factors such as growth efficiency of strains, stability of enzyme should be considered in the process of actual application.

Summerize of degradation kinetics and degradation kinetic analysis of *M. trichosporium* OB3b

Degradation kinetics of chlorinated hydrocarbon by *M. trichosporium* OB3b: Difference in strains, substrates and reaction conditions led to the otherness in the process of chlorinated hydrocarbons degradation. So kinetic characteristics of degradation of chlorinated hydrocarbons *via* the model strain *Methylosinus trichosporium* OB3b was summarized in this review. As shown in Table-1, the kinetics of the degradation of trichloroethylene (TCE) and seven other chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b were studied by Oldenhuis *et al.*[52], compounds that were readily degraded included chloroform, *trans*-1,2-dichloroethylene and trichloroethylene, with ν_{max} values of 550, 330 and 290 nmol min^{-1} mg of cells-1, respectively. Dichloromethane and chloroform were well degraded by *M. trichosporium* OB3b in the presence of soluble methane monooxygenase (sMMO) in a batch test and the rate constant of chloroform was found to be eight times higher than that of trichloroethylene, but 1,2-DCA, TCA and 1,1-DCE turned out to be poorly degraded hydrocarbons (the pseudo rate constants of these hydrocarbons were less than 2 mg/L), which demonstrated that high-affinity of soluble methane monooxygenase for chloroform was observed.

Hylckamav *et al.*[18] developed a rapid and accurate method for the determination of transformation kinetics of volatile organic substrates. It shown the degradation rates of DCM and VC were one order of magnitude higher than the other six compounds, Apparent specific first-order rate constants for cells expressing soluble methane monooxygenase decreased in the order of dichloromethane, vinyl chloride, *cis*-1,2-dichloroethene, *trans*-1,2-dichloroethene, 1,1-dichloroethene, trichloroethene, chloroform and 1,2-dichloroethane. This was the first investigation on the rate constant of VC using *M. trichosporium* OB3b.

Speitel *et al.*[17] also investigated the chlorinated aliphatic hydrocarbons degradation using the same culture, the results shown that the rate constant of chloroform (0.2-0.4 mg/d) was 2.5-11 times lower than that of trichloroethylene (0.5-3.31 mg/d) and the presence of methane caused significant enzyme competition at methane concentrations as low as 0.35 mg/L, resulting in smaller chloroform rate constants, as several researchers have reported a faster degradation of chloroform compared to that of trichloroethylene[52,53], which indicated that it was inevitable that different experimental results occurred on various condition.

Fox *et al.*[44] studied the biokinetic constants of chlorinated aliphatic hydrocarbons by adding additional enzymes including hydroxylase, reductase and NADH and the degradation rates of 1,1-DCE, *cis*-1,2-DCE and trichloroethylene were more than five orders of magnitude higher than other reported values and that of *trans* 1,2-DCE was four orders higher. Thus it was effective means that activity of soluble methane monooxygenase was enhanced by adding additional synthesis bio-enzyme. Arvin[54] found that *trans* 1,2-DCE was biodegraded quickly with mixed methanotrophs other than *M. trichosporium* OB3b by bio-membrane reactor and interestingly, the biodegradation rate of TCA was within the same order of magnitude as that of trichloroethylene.

The mutant methanotroph, *Methylosinus trichosporium* OB3b PP358 was used to study the degradation kinetics of individual chlorinated solvents and binary solvent mixtures by Aziz *et al.*[55] and Fitch *et al.*[56]. *M. trichosporium* OB3b PP358 degraded trichloroethylene (TCE), chloroform, *cis*-1,2-dichloroethylene (*c*-DCE), *trans*-1,2-dichloroethylene (*t*-DCE) and 1,1-dichloroethylene (1,1-DCE) rapidly, with maximum substrate transformation rates of > 20.8, 3.1, 9.5, 24.8 and > 7.5 mg/mg-day, respectively and half-saturation coefficients ranged from 1 to greater than 10 mg/L. No competition was observed at any of these concentrations, binary mixtures of 0.3- > 0.5 mg/L trichloroethylene with up to 5 mg/L c-DCE and up to 7 mg/L 1,1,1-TCA. Organic pollutants was complex and volatile in the wild, thus the development of degradation kinetic model of individual, binary or multiple chlorinated solvents was very necessary for meeting the application on project.

Comparing on kinetics parameter of chloralkane and chloroalkene: Kinetic constant of chlorinated hydrocarbons were significantly different. It was obvious that the half velocity constant, maximum utilization rate V_{max} and pseudo first-order rate constant k_a ranged from 0.34 to 28.6 mg/L, 0.45 to 94.2 mg/(mg_{cell} d) and 0.16-11.52 L/(mg d), respectively. It was not easy to compare the biokinetic constants of each chlorinated hydrocarbon under different cultures, substrates and

biomass conditions, when the concentration range 0.0263 to 32.9 mg/L, the maximum of V_{max} was 120 times the minimum and the maximum V_{max} and k_a of chloroform was 33.6 and 77.7 times the minimum, respectively. So types and structure of chlorinated hydrocarbon, condition of culture and activity of cells were the important influence factors on the study of biodegradation.

As a result of comparison, *M. trichosporium* OB3b was usually found to be a more efficient biodegradation culture than mixed methanotrophs. It has also been found that the extent of chlorination affects the relative rate constants of chlorinated hydrocarbon, which decreased according to the extent of chlorination. The rate constant of DCM and VC was usually higher than others at the same condition. The biodegradation rates of chlorinated alkanes were found to be significantly different from those of chlorinated alkenes. Maximum utilization rate of chlorinated alkenes was higher than that of chlorinated alkanes and that of chlorinated alkenes increased with the extent of chlorination, which was demonstrated that oxidation of double bond was easy compared with single bond (Fig. 3).

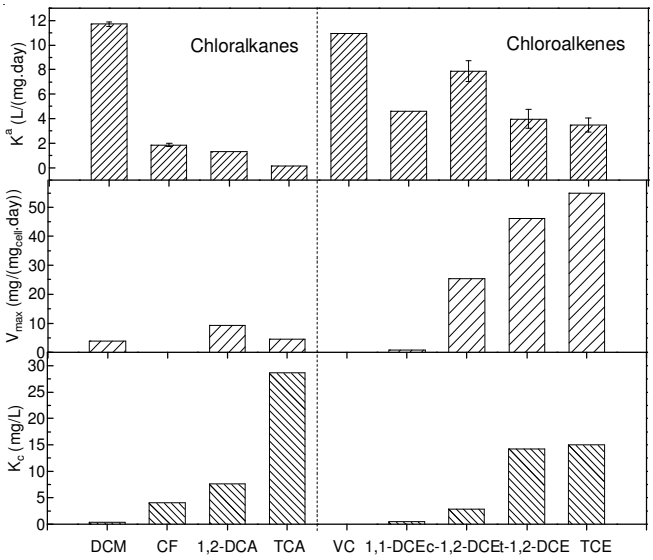

Fig. 3. Impact of pseudo first-order rate constant, maximum utilization rate and half velocity constant on the extent of chlorinate. Where DCM, chloroform, 1,2-DCA and TCA are chloralkanes and VC, 1,1-DCE, c-1,2-DCE, t-1,2-DCE and trichloroethylene are chloroalkenes

Degradation of chlorinated hydrocarbon by facultative methanotrophs: Facultative methanotrophs have unique physiological and substrate selectivity characteristic. *Methylocella* was the facultative methanotroph that was first confirmed and recognized[5]. Most of methanotrophs possess particulate methane monooxygenase and few can express both soluble methane monooxygenase and pMMO, but *Methylocella* only express soluble methane monooxygenase and lack of the system of intracytoplasmic membranes compared with public methanotrophs. On the other hand, *Methylocella* can priori use multi-carbon compounds (acetate, pyruvate, succinate, malate and ethanol).

Although the use of degradation kinetics model can help determine which methane monooxygenase should be utilized for pollutant degradation, the fact that pollutant(s) compete with the growth substrate. Methanotroph can degrade a wide range of substrates, but the fact that methane is sparingly soluble in water, can make methanotrophic-mediated bioremediation

TABLE-2
KINETIC PARAMETERS OF DEGRADATION OF CHLORINATED HYDROCARBONS BY *M. trichosporium* OB3b UNDER VARIOUS CONDITIONS (* WERE KINETIC OF DEGRADATION *via M. trichosporium* OB3b pp358)

Chlorinated hydrocarbons	Initial concentration (mg/L)	Half velocity constant K_c (mg/L)	Maximum utilization rate V_{max} (mg/(mg$_{cell}$ day))	Pseudo first-order rate constant K_a (L/(mg day))	Temperature (°C)	Reference
DCM	0.42-21.2	0.34	4.0	11.9	30	52
	-	-	-	11.52	30	18
CF	0.6-29.9	4.06	94.2	23.2	30	52
	0.1	-	-	0.3	Room	17
	0.13-0.15 (*)	3.1	2.8	0.88	22	55
	-	-	-	1.87	30	18
1,2-DCA	0.5-24.8	7.6	9.3	1.2	30	52
-	-	-	-	1.44	30	18
TCA	0.67-33.4	28.6	4.6	0.16	30	52
VC	-	-	-	10.94	30	18
1,1-DCE	0.48-24.2	0.48	0.84	1.7	30	52
	0.01-3.4 (*)	>3.4	>7.5	2.4	22	55
	-	-	-	4.61	30	18
c-1,2-DCE	0.48-24.2	2.9	25.4	8.7	30	52
	0.05-5.6 (*)	1.1	9.5	5.1	22	55
	-	-	-	7.06	30	18
t-1,2-DCE	0.48-24.2	14.3	46.2	3.2	30	52
	0.05-37 (*)	6.4	24.8	3.7	22	55
	-	-	-	4.75	30	18
	-	-	-	4.46	30	18
TCE	0.66-32.9	19.1	54.9	2.9	30	52
	0.06-8.0	10.8	-	1.7	22	55
	1.0	-	-	3.31	Room	17
	0.0263-13.15	-	-	3.08	30	13
	-	7.0-36 uM	0.454-0.757	-	30	11

challenging. Some studies have found that some, but not all facultative methanotrophs constitutively express the methane monooxygenase regardless of the growth substrate. As a result, it may be possible to utilize methanotrophs for pollutant degradation without the need for the provision of methane and thus minimizing competition for binding to the methane monooxygenase

Study on degradation of chlorinated hydrocarbons by facultative methanotroph *Methylocystis* strain SB2: *Methylocystis* strain SB2 was one of the most interesting α-Proteobacteria facultative methanotroph which was studied in recent years. It was examined for its ability to degrade chlorinated hydrocarbons when grown on methane or ethanol and was found to be constitutively expressed in the absence of methane when the strain was grown on either acetate or ethanol[25,57]. Im and Semrau[25] found that strain SB2 grown on methane degraded many chlorinated hydrocarbons like chloride (VC), *trans*-dichloroethylene (*t*-DCE), trichloroethylene (TCE) *etc*., but not dichloromethane (DCM) and all the chlorinated hydrocarbons affected the oxidation of methane. With the exception of 1,1,1-TCA, the growth of strain SB2 on ethanol was not affected by any individual chlorinated hydrocarbon. No degradation of any chlorinated hydrocarbon was observed when acetylene was added to ethanol-grown cultures, when mixtures of chlorinated alkenes or alkenes were added to cultures growing on methane or ethanol, only chlorinated alkenes degradation occurred. Collectively, these data indicate that competitive inhibition of pMMO activity limits methanotrophic growth and pollutant degradation.

Real-time quantitative polymerase chain reaction (PCR) and reverse transcription-PCR showed that the expression of pmoA decreased by one to two orders of magnitude when grown on acetate as compared with growth of strain SB2 on methane, which was studied by Yoon *et al*.[57]. Then the ability to degrade VC and *t*-DCE was lost when acetylene was added, confirming the same result by Im and Semrau[25]. Two points were summarized with the study on degradation of chlorinated hydrocarbon by Jagadevan and Semrau[16], ethanol can be used as an alternative growth substrate for promoting pollutant degradation by *Methylocystis* strain SB2 as the pMMO is not required for its growth on ethanol and ethanol can be used to enhance both pollutant transport and biodegradation by *Methylocystis* strain SB2.

Study on degradation of chlorinated hydrocarbons by facultative methanotroph *Methylomicrobium album* BG8: In the end of the 20th century, degradation of chlorinated hydrocarbons *via Methylomicrobium album* BG8 have been studied by Han and Semrau[58], Han Jong-In *et al*.[59] and Lontoh *et al*.[12] systematacially, including the study on metabolic by isotopic tracer method and degradation kinetics of chlorinated hydrocarbons. The degradation kinetics of ten halogenated hydrocarbons by *Methylomicrobium album* BG8 expressing particulate methane monooxygenase (pMMO) and the inhibitory effects of these compounds on microbial growth and whole-cell pMMO activity were measured by Han *et al*.[58]. Found that DCM, DBM, VC, *trans*-DCE, *cis*-DCE, 1,1-DCE and trichloroethylene were degraded and followed Michaelis-Menten kinetics. Finally based on these results, the compounds could be separated into four general categories, namely (1) biodegradable with minimal inactivation, (2) biodegradable with substantial inactivation, (3) not biodegradable with minimal inactivation and (4) not biodegradable but substantial inactivation of cell activity. Toxicity of trichloroethylene for cells was studied by Oldenhuis *et al*.[52] specially and the activity of cells and degradation of trichloroethylene was measured by adsorption and desorption of trichloroethylene using activated carbon. The degree of inactivation was proportional to the amount of trichloroethylene degraded. In addition, during conversion of [^{14}C] trichloroethylene, various proteins became radiolabeled, including the a-subunit of the hydroxylase component of soluble methane monooxygenase. This indicated that trichloroethylene-mediated inactivation of cells was caused by nonspecific covalent binding of degradation products to cellular proteins. Studies were performed to determine if the growth of *Methylomicrobium album* BG8 on methanol could be enhanced through the provision of chloromethane by Han *et al*.[59] and apparent rate constant K_s and maximum degradation rate V_{max} of chloromethane was 11 ± 3 μmol/L and 15 ± 0.6 nmol/(mg_{cell} min), respectively. Besides, experiments with purified pMMO from *Methylococcus capsulatus* bath showed that trichloroethylene could be mineralized to CO_2 by pMMO which was studied by Lontoh *et al*.[12] and these studies verified that pMMO was responsible for the oxidation based on acetylene inactivation studies, that propose a pathway of trichloroethylene oxidation by pMMO-expressing cells in which trichloroethylene is first converted to trichloroethylene-epoxide. The epoxide then spontaneously undergoes HCl elimination to form glyoxylate which can be further oxidized by pMMO to formate and CO_2. Alvarez-Cohen *et al*.[53] also proposed degradation pathway of trichloroethylene as shown in Fig. 4.

Tolerance and degradation kinetic of chlorinated hydrocarbon screened from aged-refuse: An novel facultative methanotroph *Methylocystis* strain JTA1 was isolated by Zhao *et al*.[60], which not only effectively enhance methane oxidation capacity of biocovers, but have high tolerance to chlorinated hydrocarbons such as trichloroethylene and chloroform. As shown in Fig. 5, at low concentration (20-50 mg/L) of chloroform, signifying that chloroform can enhance the growth of strain JTA1 once it grew on methane. Moreover, compared with growing cell, resting cell shown high activity for trichloroethylene and chloroform[60] as agreed with the study that resting cell of *M. trichosporium* OB3b can degrade chloroform completely[44]. Meanwhile, *Methylocystis strains* were enriched from aged refuse and the degradation of trichloroethylene by

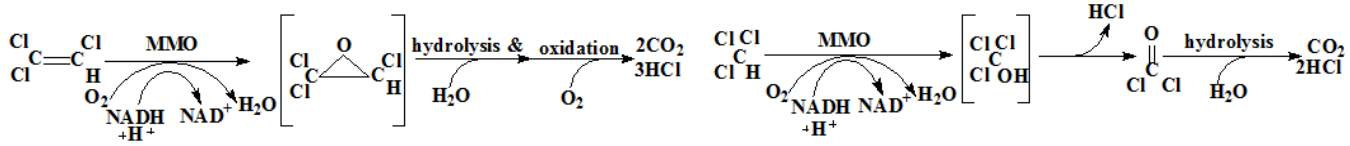

Fig. 4. Process of degradation of trichloroethylene and chloroform by MMO [Ref. 53]

its resting cells were studied and the kinetic equation of trichloroethylene degradation by methanotrophs community was also fitted which fitted to Monad model well, as shown in Fig. 6. The degradation rate reached 79 % when the initial concentration of trichloroethylene was 45.5 mg/L and cells concentration C_x was 1.728 g/L. The maximum specific degradation rate was $q_{s,\ max} = 1.51 \times 10^{-4}$ min^{-1} and half-saturation constants (K_S = 2.58 mg/L, R^2 = 0.961) which was much lower than that of reported methanotroph *Methylosinus trichosporium* OB3b (19.1 mg L^{-1}), indicating that the *Methylocystis strains* had higher tolerance and affinity potential to trichloroethylene. These study extended the application field of facultative methanotrophs effectively, carrying out the study of methanotrophs was hopeful to achieve new breakthrough.

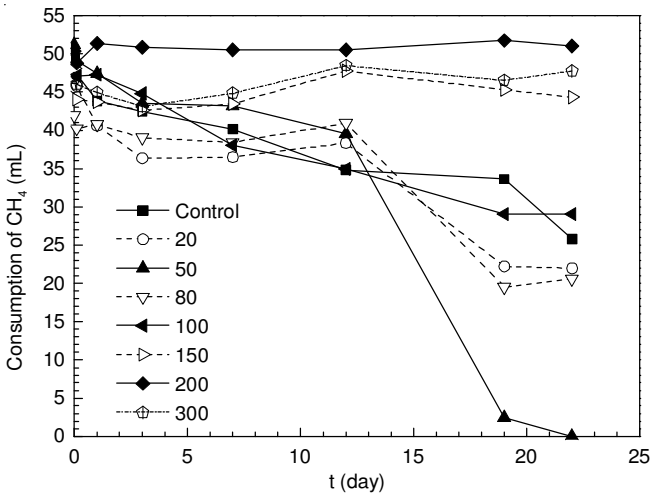

Fig. 5. Chloroform tolerance of *Methylocystis* strain JTA1 at different concentrations of chloroform [Ref. 60]

Catalytic property and utilization potentiality of pMMO: Particulate and soluble methane monooxygenase differ not only in their cellular location but also in their sensitivity to inhibitors, with the particulate form being the more susceptible enzyme[61], Also, the pMMO substrate range is narrower than that of the soluble form of the enzyme and the rate of degradation was lower, therefore, much of the early literature focused on the utility of soluble methane monooxygenase-expressing aerobic methanotrophs for pollutant degradation and these studies typically focused on simple systems where one methanotrophic culture (either pure or mixed) was exposed to one pollutant. It suggested that pMMO-expressing aerobic methanotrophs may be prefer able as these cells are able to survive more easily in the presence of relatively complex mixtures of chlorinated ethenes that are more representative of what could be found *in situ*[46]. pMMO can oxidize alkanes and alkenes up to five carbon atoms in length, but not cyclic or aromatic compounds[42,49,62]. In addition, some studies have shown that pMMO is energetically more favorable than that using soluble methane monooxygenase, with carbon conversion efficiencies being up to 38 % higher than cells expressing the soluble form of the enzyme[63], which suggested that pMMO-expressing aerobic methanotrophs possess broader carbon sources when was in condition of biodegradation, thus made it possible that overcoming the fact that

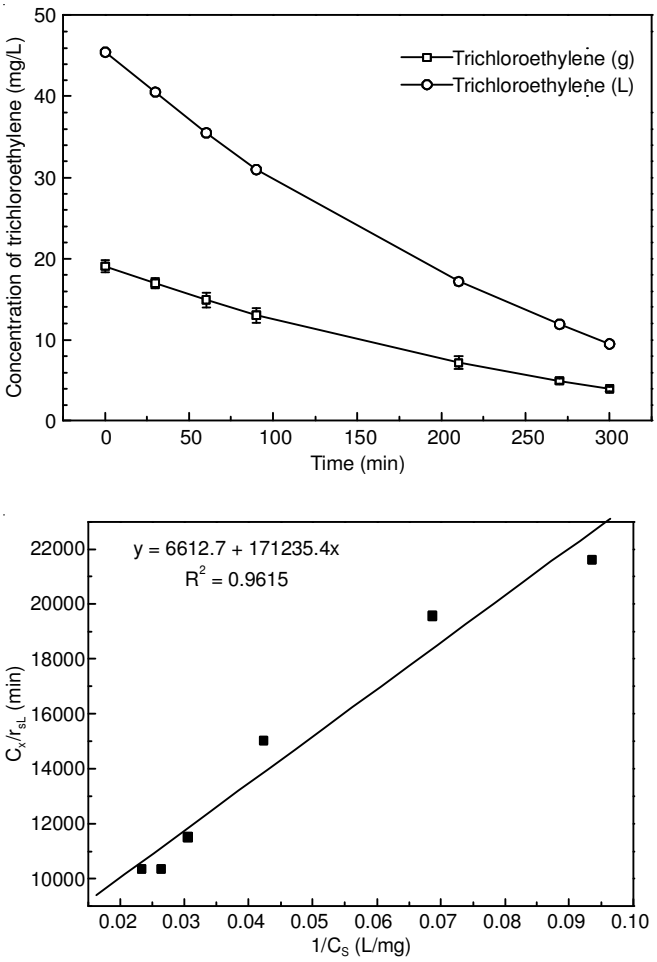

Fig. 6. Degradation curve of trichloroethylene by methanotrophs and degradation kinetics of trichloroethylene based on monad equation. Where C_x, r_{sL} and C_S are dry weight of the strain, average degradation rate of trichloroethylene in the interval time of measure and concentrations of trichloroethylene, respectively

methane is sparingly soluble in water by adding multi-carbon compounds such as acetate. The study on degradation of trichloroethylene and tetrachloroethylene by flushing on ethanol *in situ* have been carried out and agreed with the feasibility[64,65]. From these observations, a simple model, the "ΔModel" was developed based on Michaelis-Menten kinetics to predict when either soluble methane monooxygenase or pMMO-expressing systems would be preferred by Lee *et al.*[46] and Yoon *et al.*[66]. Results that pMMO-expressing methanotrophs may be preferred over soluble methane monooxygenase-expressing methanotrophs over abroad range of pollutant concentrations, so facultative methanotrophs are advantageous in degradation of chlorinated hydrocarbon.

Facultative methanotrophs may actually generate some benefit from oxidizing these compounds. Such a phenomenon is unknown, for as described earlier, chloromethane has been found to stimulate methanotrophic growth when methanol was provided as the growth substrate[34]. It is still unclear how facultative methanotrophs assimilate carbon from multi-carbon compounds and all facultative methanotrophs to date group with the α-Proteobacteria and use the serine cycle for carbon assimilation from formaldehyde. Many acetate assimilation pathways made malate and glyoxylate, which are intermediates

of the serine cycle[14,44] and glyoxylate was primary product of trichloroethylene oxidation by pMMO-expressing methanotrophs as well as by purified soluble methane monooxygenase as well as by purified soluble methane monooxygenase[67], suggesting that for facultative methanotrophs and obligate methanotrophs that utilize the serine cycle for carbon assimilation.

Problems and future research directions for degradation of chlorinated hydrocarbons by methanotrophs: In the past 20 years, much substantial progress was achieved on application of chlorinated hydrocarbon biodegradation by methanotrophs, including bioremediation of chlorinated hydrocarbons in bioreactor[20,56,68-76] and biological removal of contaminant *in situ*[19,77-80]. *ex situ* Bioremediation methods using bioreactors for the removal of organic contaminants have two forms of configurations, single-stage which growth on the primary substrate (methane) and degradation of contaminants occur in one reactor and multi-stage. Advantage of multi-stage bioreactor was avoided the competitive inhibition between the growth substrate and contaminants and then enhanced the ability of biodegradation. The critical process of bio-remediation was stimulating local mixed methanotrophs by adding safe and economical carbon source and nitrogen source *in situ*.

Although methanotrophs show broad application prospect on bioremediation of pollutant(s), the isolated strains capable of degrade chlorinated hydrocarbons and easy control was limited and the study on molecular level was deficient for methanotrophs. Furthermore, obligate methanotrophs only use methane or methyl as carbon source, which made it difficult in enriching cells on a large scale. The competitive inhibition between growth substrate and chlorinated hydrocarbons, among the chlorinated hydrocarbons was exist on progress of biodegradation by facultative methanotrophs [47,53,55]. Also, toxicity of pollutant(s) and co-metabolic product led to low activity of cells and efficiency of degradation[44, 81].

Future work should determine the abundance and distribution of facultative methanotrophs *in situ* using high throughput sequencing and metagenomic analysis widely. Study on facultative methanotrophs only take a small step and the public facultative methanotrophs were less than 10[14,82,83]. So research on separation and purification, biological specificity for more novel strains and more novel strains was urgent. Then degradation kinetic should be performed, including cell growth kinetics, degradation of chlorinated hydrocarbons and inhibition kinetics of co-metabolic substrate. Degradation mechanism of chlorinated hydrocarbons and other persistent organic pollutants would be revealed by deducing kinetic parameter. Finally, developing new bioreactor and strengthening cell growth were also critical step to ensure engineering application. These studies would provide theoretical guidance and foundations in microbiology for bioremediation of chlorinated hydrocarbons.

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