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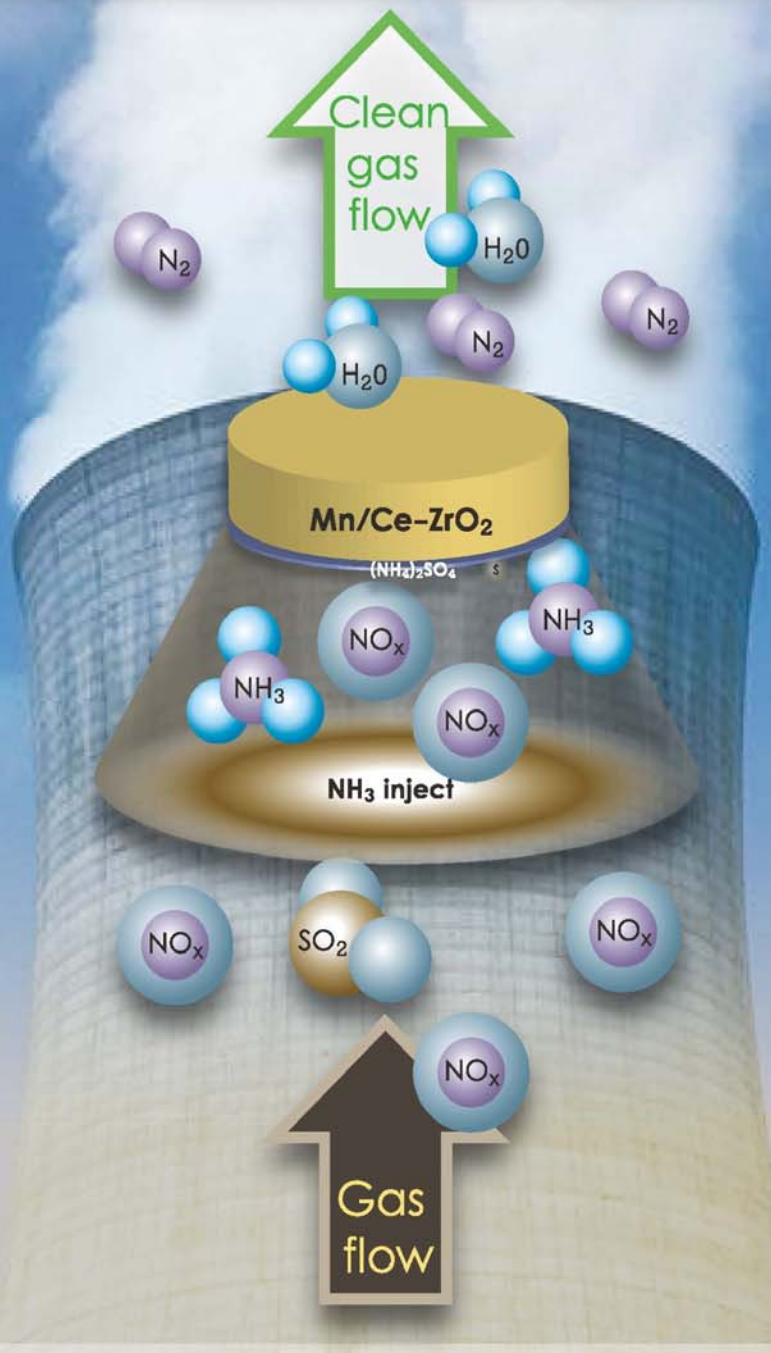

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Characterization of *Methylocystis* strain JTA1 isolated from aged refuse and its tolerance to chloroform

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Abstract

To accelerate the efficiency of methane biodegradation in landfills, a Gram-negative, rod-shaped, non-motile, non-spore-forming bacterium, JTA1, which can utilize methane as well as acetate, was isolated from the Laogang MSW landfills, Shanghai, China. Strain JTA1 was a member of genus *Methylocystis* on the basis of 16S rRNA and *pmoA* gene sequence similarity. The maximum specific cell growth rates (μ_{max}= 0.042 hr^{-1}, R^2= 0.995) was derived through Boltzmann simulation, and the apparent half-saturation constants ($K_{m(app)}$ = 7.08 mmol/L, R^2 = 0.982) was calculated according to Michaelis-Menton hyperbolic model, indicating that *Methylocystis* strain JTA1 had higher-affinity potential for methane oxidation than other reported methanotrophs. By way of adding the strain JTA1 culture, the methane consumption of aged refuse reached 115 mL, almost two times of control experiment. In addition, high tolerance of *Methylocystis* strain JTA1 to chloroform could facilitate the methane oxidation of aged refuse bio-covers. At the chloroform concentration of 50 mg/L, the methane-oxidation rate of bio-cover reached 0.114 mL/(day·g), much higher than the highest rate, 0.0135 mL/(day·g), of reported bio-covers. In conclusion, strain JTA1 opens up a new possibility for environmental biotechnology, such as soil or landfills bioremediation and wastewater decontamination.

Key words: methane; aged refuse; *Methylocystis*; facultative methanotrophs; chloroform tolerance
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Introduction

Methane (CH_4) is a relatively potent greenhouse gas, contributing to around 20% of the global warming (Howarth et al., 2011). Methane in landfill gas is a significant source of human-caused greenhouse gas, accounting for about 25% of annual global human-caused methane emissions (Vigliotta et al., 2007). In China, the disposal of municipal solid waste (MSW) by landfilling in 2008 has reached approximately 85.59 million tons, thereby releasing more CH_4 than western countries since food waste account for more than 55% of MSW (Scheutz et al., 2004). Therefore, the reduction of methane emissions is critical to maintain the methane balance.

Methanotrophs in MSW can exert an important role in reducing methane emissions (Conrad, 2007; Zhao et al., 2010). In general, most aerobic methanotrophs take methane as their unique carbon and energy source, together with some other one-carbon compounds, thereby being termed as "obligate methanotrophs" (Conrad, 1996, 2007). On the contrary, facultative methanotrophs are capable of utilizing both methane and some multi-carbon compounds as the carbon and energy source. This can remarkably broaden the application of facultative methanotrophs to lower methane emissions (Khomyakova et al., 2011; Semrau et al., 2011). The facultative methanotrophs were recently demonstrated in several α-proteobacterial methanotrophs of the genera, such as *Methylocella*, *Methylocystis*, *Methylocapsa* and the genera *Clonothrix* and *Crenothrix* (Belova et al., 2011; Dedysh et al., 2005; Dunfield et al., 2003; Stoecker et al., 2006). Members of these genera are totally different in view of their physiology and substrate preferences. Therefore, more important thing is to isolate and culture bacteria, which have high methane-oxidizing ability to reduce the emission of human-caused methane.

However, study on isolating or culturing pure facultative methanotrophs were rarely reported in landfills according to our knowledge. In this work, a strain, named JTA1, was isolated from oligotrophic aged-refuse rested on our previous work on the landfill bio-covers, as well as identified by phylogenetic analysis based on the 16S rRNA and morphological characteristics. Furthermore, physiological characteristics of strain JTA1 were studied,

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including maximum specific cell growth rate and apparent half-saturation constant. Finally, study on strengthening methane oxidation of aged refuse via strain JTA1 and tolerance of strain JTA1 to chloroform were carried out to evaluate the potential possibility of applying the landfill bioremediation to the engineering field.

1 Materials and methods

1.1 Materials

Aged refuses were collected in 2009 from Laogang Refuse Landfill, Shanghai, the largest landfill in China. The samples of aged refuses, containing methanotrophs, were excavated from each section according to disposed periods and the disposal ages of the landfill. The finer fraction in aged refuse was applied in the experiments employing the 4 mm mesh sieve (Lou et al., 2011). The carbon substrates, chloroform (> 99%) and mineral salts for MNMS (modified nitrate mineral salts) medium were purchased from Sigma-Aldrich and Fisher.

MNMS medium contains (g/L): $NaNO_3$, 0.850; KH_2PO_4, 0.530; Na_2HPO_4, 0.170; $MgSO_4{\cdot}7H_2O$, 0.037; $CaCl_2{\cdot}2H_2O$, 0.007; and $FeSO_4{\cdot}7H_2O$, 0.011. Trace element solution was 2 mL with pH 6.8. Trace element solution contains (mg/L): $ZnSO_4{\cdot}7H_2O$, 0.204; $CuSO_4{\cdot}5H_2O$, 1.25; $MnSO_4{\cdot}4H_2O$, 0.223; H_3BO_3, 0.062; $Na_2M_OO_4{\cdot}2H_2O$, 0.048; and $C_OCl_2{\cdot}6H_2O$, 0.048.

1.2 Isolation of *Methylocystis* strain JTA1

Aged refuse contained a large and diverse population of micro-organisms with a high capacity for decomposing refractory organic matter presented in some wastewaters, including leachate, and aged refuse-based bio-covers were found as one of the effective methane-oxidizing covers for landfills (Zhao et al., 2009, 2011). Aged refuses with different ages, i.e. 8, 10 and 12 years, were inoculated into the MNMS medium, and methane was introduced to enrich the methane-oxidizing microorganisms in aged refuse. The acquired culture was then subcultured 10 times to further enrich the methanotrophs. Pure methane-oxidizing strain was finally isolated by screening single colony on MNMS agar plates.

1.3 PCR analyses

A single colony of the target strain was inoculated into MNMS medium. Mid-exponential phase cells were collected by centrifugation and genomic DNA was extracted using QIAquick Genomic DNA Buffer Set. The PCR products were electrophoresed in a 1.5% agarose gel (20 cm × 25 cm) for 4 hr at a constant voltage of 4 V/cm in TBE buffer. Then, PCR products were purified and sequenced without cloning into *Escherichia coli* Blast of the 16S rRNA gene and MMO sequences were performed on NCBI, and the phylogenetic dendrogram was built by MAGA 4.0 program. PCR amplification of 16S rRNA genes was performed using universal primers F27 (5′-AGAGTTTGATCATGGCTCAG-3′) and R1492 (5′-TACGGTTACCTTGTTACGACTT-3′), *mmoX* gene (encoding a subunit of soluble methane monooxygenase) was amplified using mmoXA (5′-ACCAAGGARCARTTCAAG-3′) and mmoXB (5′-TGGCACTCRTARCGCTC-3′), *pmoA* gene (encoding a subunit of soluble methane monooxygenase) was amplified using A189gc (5′-GGNGACTGGGACTTCTGG-3′) and mb661 (5′-CCGGMGCAACGTCYTTACC-3′).

1.4 Growth experiments of *Methylocystis* strain JTA1

Cultures were grown in 400 mL MNMS medium in 1 L Erlenmeyer flasks capped with gas-tight silicone stoppers. Methane (about 20%, *V*/*V*) was added to the headspace by using a syringe and a sterile filter (0.22 μm), and other carbon sources were added into MNMS medium to a concentration of 3 g/L. Inoculant cells were obtained from MNMS agar plates with methane as solo carbon source and were then washed three times in sterile water before inoculation. Cultures, with the inoculation amount of 5.0%, were grown at 30°C with 150 r/min shaking on a rotary shaker. Uninoculated media were used as blanks for leakage and sterility control, and inoculated MNMS medium without carbon source was included to verify that no cryptic growth occurred. Samples were taken daily to determine the $OD_{600\,nm}$ and concentrations of methane directly. The growth model of the strain JTA1 was established by introducing Boltzmann simulation:

$$y = \frac{A_1 - A_2}{1 + e^{(x-x_0)/dx}} + A_2$$

where, y and x (hr) represent $OD_{600\,nm}$ and time, respectively; A_1, A_2, d and x_0 are Boltzmann parameters and dimensionless except for x_0 (hr). The values of these parameters could be fitted by Origin 7.5, and the maximum specific cell growth rate (μ_{max}, hr^{-1}) was calculated by interpolation methods.

The 500-mL batches of the culture were grown on methane for the experiments, and 20 mL of the culture ($OD_{600\,nm}$ = 0.31) were transferred to 100-mL serum vials. Cell counts were not done because this study aimed at studying substrate affinity rather than specific activity. The vials were capped with butyl rubber stoppers and injected with methane at different ratios. Cultures were grown at 30°C with 150 r/min shaking on a rotary shaker, and methane was measured at an interval of 2 hr. Finally, chloramphenicol with the final concentration of 50 mg/L was added to deter the further enzyme production after the reactions finished. Initial reaction rates with different concentrations of methane could be calculated by linear regressions of methane consumption versus time.

1.5 Tolerance of *Methylocystis* strain JTA1 to chloroform

Because chloroform is liquid at room temperature, saturated stock solutions were prepared according to the method developed by Chang and Alvarez-Cohen (1996). Chloroform was injected at an initial concentration of 40 mmol/L. The added amount was calculated using the following dimensionless Henry's constants: 0.189 (Gossett, 1987). Distilled deionized water (> 18 MΩ·cm) from a Corning Millipore D2 system was used for all experimental setups. All glassware were washed with detergent and then soaked in 2 mol/L HNO_3 overnight to remove trace metals. Nitric acid was subsequently removed by repeated rinses with distilled deionized water. All conditions were performed in duplicate.

1.6 Analysis methods

The pure cells of strain JTA1 were freeze-dried for at least 24 hr and coated by gold under vacuum for 20 min. Then, images were obtained using the samples by environmental scanning electron microscopy (ESEM XL-30, Philips, Holand). Methane and CO_2 were analyzed by gas chromatography (Shimadzu GC-14B, Japan) with a stainless steel column packed with Carbosive SII (3.2 mm in diameter and 2.0 m in length) and thermal conductivity detector. The temperatures of injector, detector and column were kept at 100, 105 and 60°C, respectively. Nitrogen was used as the carrier gas with the flow rate of 30 mL/min. The measured volume was adjusted to the volume at the standard temperature and pressure. The optical density at 600 nm was measured by Eppendorf BioPhotometer (Hamburg, Germany).

2 Results and discussion

2.1 Physio-biochemical and phylogenetic analysis of *Methylocystis* strain JTA1

The morphology, determined by ESEM, of the freeze-dried cells of the grown-on-methane strain JTA1 is shown in **Fig. 1**. The cells were Gram-negative, non-motile, non-spore-forming and rod-shaped, with a round-bowl-shaped concave in the centre. The outer diameter of cell ranged from 0.2 to 0.4 μm, and the length ranged from 0.6 to 0.8 μm.

PCR-amplified 16S rRNA gene products, grown on methane and acetate respectively, were separated by agarose gel electrophoresis. Then, the sequences of the 16S rRNA gene of strain JTA1 (GenBank: KC129107) were determined and compared with reported facultative methanotrophs. The similarity levels of sequence between strain JTA1 and *Methylocystis* strain H2s and SB2 reached 97.13% and 97.45%, respectively, suggesting that it was a member of genus *Methylocystis*. According to the *pmoA* gene sequence, the similarity levels of strain JTA1 to

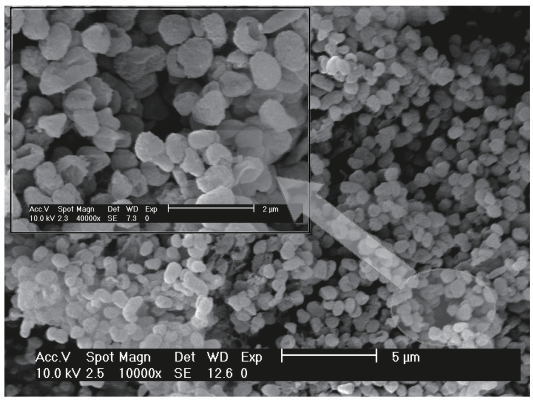

Fig. 1 SEM micrograph of *Methylocystis* strain JTA1.

Methylocystis strain H2s and SB2 were 87.84% (GenBank: FN422005) and 91.72% (GenBank: GU734137). At the optimal growth pH of 6.0–6.5, strain H2s could utilize not only methane and methanol but also acetate for growth, tending to be a mild acidophile with functional genes for both sMMO and pMMO. However, strain JTA1 could merely express pMMO, the same as strain SB2, collected from a spring bog with an optimal growth pH of 6.8 (Im and Semrau, 2011). All experimental findings suggested that strain JTA1 belongs to a new kind of facultative methanotrophs.

As shown in the phylogenetic dendrogram (**Fig. 2**), *Methylocystis* strain JTA1 and other seven reported kinds of facultative methanotrophs could be classified into two clusters. The genera *Clonothrix* and *Crenothrix* are considered to be Type I methanotrophs for they are phylogenetic subsets of the Methylococcaceae family (Op den Camp et al., 2009). It has been reported that *Crenothrix polyspora* can take up acetate, and to a lesser extent, glucose, in the absence of CH_4, suggesting that this bacterium should be a facultative methanotroph (Stoecker et al., 2006). Interestingly, another recently discovered filamentous methanotroph, *Clonothrix fusca*, closely phylogenetic related to *C. polyspora* based on 16S rRNA gene sequence, was not able to grow on glucose (Vigliotta et al., 2007). To date, however, limited research has been done to isolate and cultivate these strains in pure culture.

The optimal growth of strain JTA1 occurs at pH 6.8 and temperature 35°C. The utilization of carbon sources by strain JTA1 is shown in **Table 1**. Strain JTA1 could utilize not only methane and methanol but also acetate

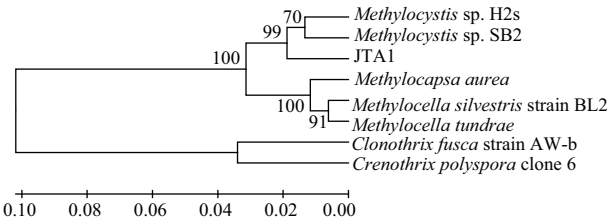

Fig. 2 Phylogenetic tree based on 16S rRNA gene sequences of the type strains of facultative methanotrophs.

Table 1 Carbon sources utilization of JTA1

Carbon source	Growth	$OD_{600\ nm}$
Methane	++	1.231 ± 0.037
Methanol	+	0.357 ± 0.017
Formate	–	0.062 ± 0.010
Formaldehyde	–	0.060 ± 0.012
Methylamine	–	0.065 ± 0.009
Urea	–	0.058 ± 0.008
Ethanol	+	0.213 ± 0.016
Oxalate	–	0.061 ± 0.013
Acetate	+	0.521 ± 0.033
Lactose	–	0.060 ± 0.005
Glucose	–	0.051 ± 0.007
Malate	–	0.053 ± 0.005

++: substantial growth; +: trace growth; –: no growth.

and ethanol for growth. It has been reported that growth of *Methylocystis* strain SB2 on methane was the highest, then on ethanol, with the maximum $OD_{600\ nm}$ of 0.83 and 0.45, respectively. Moreover, strain JTA1 grew better on methane than strain SB2, with a maximum $OD_{600\ nm}$ of 1.23, while grew less on ethanol with a maximum $OD_{600\ nm}$ of 0.21. *Methylocystis* strain JTA1 could not utilize C_5 or C_6 compounds as carbon sources, evincing that the bacteria lacked the complete citric acid cycle in γ-Proteobacteria methanotrophs (2-ketoglutarate dehydrogenase activity is missing) or failed to transfer extracellular compounds containing carbon-carbon bond such as most obligate or reported facultative methanotrophs (Wood et al., 2004).

2.2 Kinetics of methane affinity

The growth model of strain JTA1 was determined by Boltzmann simulation, and the parameters deduced from the model can well match with the investigation ones (**Fig. 3**). The maximum specific cell growth rate (μ_{max}) of strain JTA1 was calculated by interpolation methods to be 0.042 hr^{-1} (R^2 = 0.9951), which is higher than *Methylocella silvestris* (μ_{max} = 0.033 hr^{-1}) and *Methylocapsa aurea* (μ_{max}= 0.018 hr^{-1}), but lower than *Methylocystis* strain H2s (μ_{max}= 0.06 hr^{-1}) and *Methylocystis* strain SB2 (μ_{max}= 0.052 hr^{-1}) (Semrau et al., 2011). These data indicated that methane is the preferred substrate for strain JTA1 growth when compared with other facultative methanotrophs.

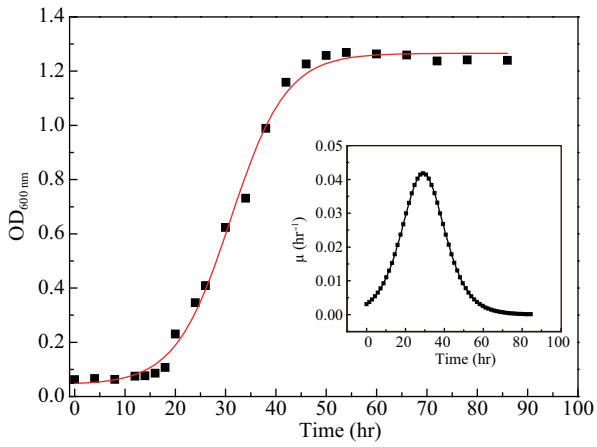

Fig. 3 Growth curve of strain JTA1 fitted by Boltzmann model. The curve shows the growth of *Methylocystis* strain JTA1 utilizing methane as the sole carbon and energy source. Each symbol represents the mean of results with two samples.

The kinetic curves of strain JTA1, grown on different initial methane concentrations, are demonstrated in **Fig. 4**. The reaction rates of methane at initial concentrations from 1.6 to 40.9 mmol/L was modeled by linear regressions of methane consumption versus time, with R^2 from 0.9349 to 0.9958. The initial reaction rates were plotted against the methane concentrations and fitted to a Michaelis-Menton hyperbolic model. The apparent half-saturation constants $K_{m(app)}$ was derived through Lineweaver-Burk methods and the value was 7.08 mmol/L (R^2= 0.982), indicating that strain JTA1 had higher-affinity potential for methane oxidation compared with the reported methanotrophs (Dunfield et al., 1999).

2.3 Strengthening *ex situ* methane oxidation of aged refuse via strain JTA1

It has been proved that landfill bio-covers can effectively mitigate the emission of greenhouse gas from landfill. Also, bio-covers have also been listed in the Intergovermental Panel on Climate Change working Group III assessment report (Scheutz et al., 2004), and aged refuse might be one of the most promising materials for alleviating the CH_4 emissions according to our previous publication (Lou et al., 2011). In this work, strain JTA1 culture was sprayed to facilitate the methane-oxidation rate of aged refuse. As shown in **Fig. 5**, the logarithmic phase started at day 5 and the methane-oxidation rate increased steeply after adding 1 mL strain JTA1 culture with the $OD_{600\ nm}$ of 1.01 into 20 g aged refuse. The final methane consumption reached 115 mL, almost two times of control experiment (61 mL).

2.4 Tolerance of *Methylocystis* strain JTA1 to chloroform

It has been found that chloroform could effectively inhibit the metabolic process of methanogenesis. The practice of

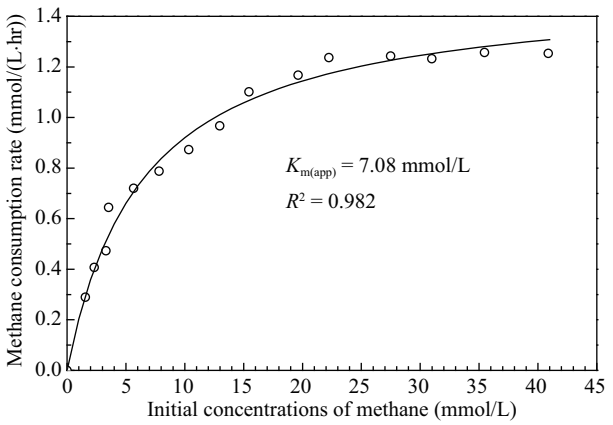

Fig. 4 Kinetic curve of methane oxidation grown on different initial concentrations of methane. Each symbol represents the mean of results with two samples.

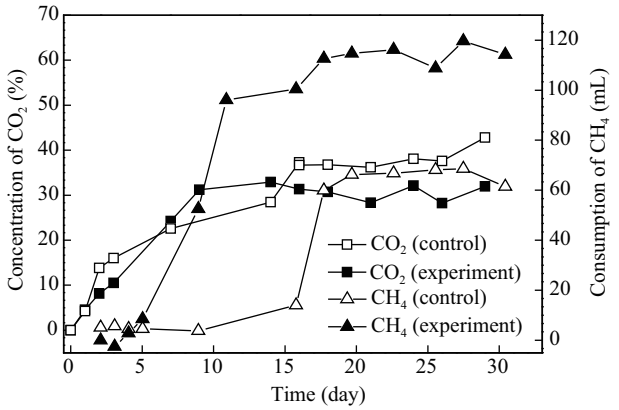

Fig. 5 Effects of adding JTA1 culture on methane oxidation in aged refuse.

inhibition technology on landfills was feasible to reduce the emissions of human-caused CH_4 from landfills according to our previous publication (Zhao et al., 2009). In this work, the tolerance of *Methylocystis* strain JTA1 to chloroform was studied to explore the feasibility of integrated technology with both bioinhibiton of methanogenesis and bioaugmentation of methanotrophs. After adding 1 mL strain JTA1 culture with $OD_{600\,nm}$ of 1.05, different concentrations of chloroform were added into 20 g aged refuse. As shown in **Fig. 6**, the activity of strain JTA1, compared with control experiment, was strengthened when chloroform concentration was less than 80 mg/L. In particular, the removal rate of CH_4 reached 100% after 22 days when the concentration of chloroform was 50 mg/L, signifying that chloroform can enhance the growth of strain JTA1 once it grew on methane as *Methylomicrobium album* BG8 (Han and Semrau, 2000). However, the inactivation of cells occurred when the concentration of chloroform was more than 200 mg/L. Under this scenario, the bioinhibiton effect of chloroform was dominant.

At the chloroform concentration of 50 mg/L, the methane-oxidation rate of bio-cover reached 0.114 mL/(day·g), which is much higher than that of reported aged refuse (0.0068–0.0135 mL/(day·g)) (De Visscher et al., 1999). In other words, high chloroform tolerance of strain JTA1 could be not only conductive to the methane oxidation, but also suitable for the integrated technology to reduce the emission of methane from the landfills.

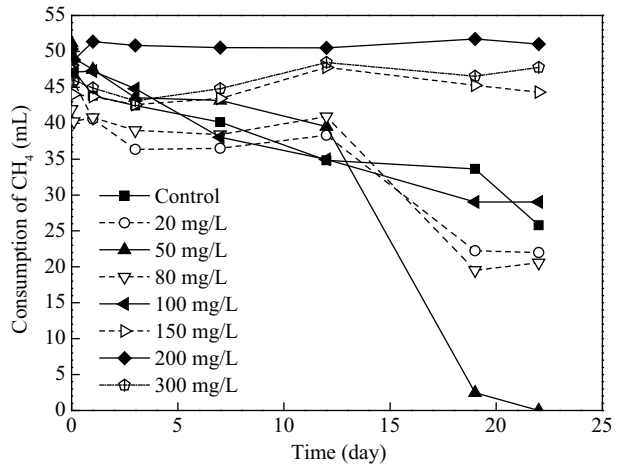

Fig. 6 Chloroform tolerance of *Methylocystis* strain JTA1 at different concentrations of chloroform.

3 Conclusions

Methylocystis strain JTA1 isolated from oligotrophic aged-refuse has great tolerance to chloroform, thereby helping it through early bioinhibiton operation on landfill as well as maintaining its activity. *Methylocystis* strain JTA1 can utilize methane as well asacetate, and high cell density with the $OD_{600\,nm}$ of 1.1–1.2 was achieved with methane as the carbon source. Moreover, the value of $K_{m(app)}$ was 7.08 mmol/L, indicating that strain JTA1 had higher-affinity potential for methane oxidation than other reported methanotrophs. Adding strain JTA1 could dramatically increase methane-oxidation rate of aged refuse, which is conducive to applying obligate methanotrophs to the engineering field. In conclusion, *Methylocystis* strain JTA1 opens up a new possibility for environmental biotechnology, such as soil or landfills bioremediation and wastewater decontamination.

Acknowledgments

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