Korean J. Chem. Eng., **30**(9), 1770-1774 (2013)
DOI: 10.1007/s11814-013-0099-3

Growth kinetics of an indigenous mixed microbial consortium during methylene chloride degradation in a batch reactor

Rajamanickam Ravi*,†, Ligy Philip, and Tyagarajan Swaminathan*****

*Department of Chemical Engineering, Annamalai University, Chidambaram, Tamil Nadu-608001, India
**Department of Civil Engineering, Indian Institute of Technology Madras, India
***Department of Chemical Engineering, Indian Institute of Technology Madras, India
(*Received 22 January 2013 • accepted 30 May 2013*)

Abstract−Biodegradation of methylene chloride by a mixed microbial culture, isolated from a common sewage treatment plant, was investigated in a batch system. Batch experiments were performed at room temperature (27 °C) and pH value of 7. The methylene chloride concentration in growth media varied from 25 mgl^{-1} to 250 mgl^{-1}. A maximum observed degradation was 1 $mgl^{-1}h^{-1}$ at 100 mgl^{-1} of methylene chloride. The culture followed substrate inhibition kinetics and specific growth rate were fitted to different substrate inhibition models (Haldane, Aiba and Edwards models) by MATLAB 7.1@. Among all models, Haldane was found to better fit with root mean square of 0.947. The biokinetic constants estimated using these models show good potential of the mixed microbial culture in methylene Chloride degradation. *Escherichia coli* and *Staphylococcus aureus* are predominant microbes present in the mixed culture.

Key words: Mixed Culture, Dichloromethane, Biodegradation, Kinetics, Inhibition Models

INTRODUCTION

The Clean Air Act Amendments (CAAA) of 1990 proposed by the United States Environmental Protection Agency (US-EPA) distinguishes 188 air pollutants based on their inherent toxicity. One VOC of particular interest is methylene chloride, which is a common solvent used by chemical and process industries, primarily for metal degreasing, paint removal, finishing solvent in electronics manufacturing, degreasing agent for citrus fruit and removal of caffeine from coffee (EPA, USA). Methylene chloride exists widely in aqueous environment due to its relatively high water solubility (20 gl^{-1} at 20 °C). With a low boiling point (40.1 °C) and high stream pressure (47 kpa at 20 °C) methylene chloride also enters the environment via gaseous emissions. It currently is regarded as a kind of environmental organic pollutant with high toxicity. Apparently, methylene chloride elimination technology becomes necessary for environmental protection [1,2]. Acute inhalation exposure to high levels of methylene chloride can affect the central nervous systems (CNS) of humans with symptoms like decreased visual, auditory and psychomotor functions, but these effects are reversible once exposure is reduced [3,4].

Methylene chloride is a highly toxic substance with carcinogenic and mutagenic effects. At low concentration (10 mgl^{-1}) it has the capacity to disturb biological processes in human bodies [5]. It is a major pollutant in wastewaters and communal waters despite the efforts made to decrease its production [6]. Hence, treatment/removal of such methylene chloride is essential to prevent deterioration of ecosystem.

There are different methods for the treatment of methylene chloride present in water/Air. The most common approach is either chemical or biological treatment. Chemical treatment focuses on advanced oxidative processes (AOPs), while biological transformation utilizes both bacterial and fungal systems. Both anaerobic and aerobic biodegradation processes have been investigated for the biodegradation of methylene chloride [1,7]. Biological treatment is especially effective in the lower concentration range and relatively cheaper as compared to other traditional methods [8]. One major problem with the bacterial degradation process is the enzyme inhibition due to increasing concentration of degraded product, a classic example of feedback inhibition [9]. Moreover, high concentration of methylene chloride may cause irreversible cell damage, and inhibit the activity of coenzyme regeneration cycle and the carbon source facilitation [10]. VOC (methylene chloride) can serve as energy and or carbon sources for the microbial metabolism [11].

The aim of the study is to evaluate the removal of methylene chloride using acclimatized mixed culture in a batch reactor and indentify the predominant microorganism present in the mixed culture.

MATERIALS AND METHODS

1. Microorganism and Culture Media Used

Methylene chloride degrading mixed microbial culture was isolated and enriched from a municipal sewage treatment plant located in Chennai, India. The culture was initially grown in a 250 ml Erlenmeyer flask containing 100 ml of mineral salt medium (MSM) containing (mgl^{-1}) : KH_2PO_4-2, $MgSO_4 \cdot 7H_2O$-0.025, $(NH_4)_2SO_4$-2.0, NaCl-0.5 and glucose 1 gl^{-1} in distilled water. The pH of the mineral salt media was adjusted to 7. Cultures were acclimatized to MSM containing methylene chloride (10 mgl^{-1}) as the sole carbon source for a period of three weeks to grow.

2. Batch Biodegradation Study

All the biodegradation experiments performed using the mixed culture were performed in a 250 ml Erlenmeyer flask, 100 ml of MSM containing methylene chloride at the concentration range of 25 mgl^{-1} to 250 mgl^{-1} is used. Upon incubations of the flask at 27 °C, samples were withdrawn at regular intervals, centrifuged (10,000 ×g

†To whom correspondence should be addressed.
E-mail: lect_ravi@yahoo.com

for 5 mins) and analyzed for residual methylene chloride concentration. For each concentration, duplicate experiments were carried out for period of up to 48 h or until the residual concentration in the flask was found to saturate.

3. Analytical Methods

The methylene chloride content in biomass free samples was determined using gas chromatography equipped with flame ionization detector (Model 5765, Nucon gas chromatograph, Nucon Eng. India) and Poropak column (1/8" ID, liquid - 10% FFAP, solid - Ch-WIHP, 80/100 mesh). Nitrogen was used as the carrier gas. The temperatures of the injection port, oven and detection port were 150, 120 and 250 °C, respectively. The flow rate of elutant was kept at 1 ml min^{-1}. Biomass concentration in the samples was monitored by measuring the absorbance at 600 nm using UV-visible spectrophotometer [Lambda 2.5, Perkin-Elmer,USA].

4. Identification of Isolates

Biochemical identification of microbes was carried out using standard biochemical tests and microscopic observations. The isolates were identified based on morphological observation and biochemical characterization. The tests involved were gram staining, amylase production, citrate utilization, indole test etc. [12]. Bergey's Manual of Determinative Bacteriology was used as a reference to identify the isolates [13].

For the isolation and identification of *E. coli*, the enriched sample was cultured on selective medium Levine Eosin Methylene Blue (EMB) Agar and incubated at 37 °C for 24 h. Morphologically typical colonies (at least four/plate) producing metallic sheen were taken into nutrient broth for further identification. *S. aureus* was isolated using the technique given by Baird Parker. Enriched samples were streaked on Baird Parker Agar (BPA) and the plate was incubated at 37 °C for 24-48 h. Appearances of jet-black colonies surrounded by white halo were considered presumptive of *S. aureus*.

5. Kinetic Experiment

A colony of strain from Agar plate was transferred to sterile nutrient medium in 100 ml Erlenmeyer flask. The flask was incubated for 10 h at 27 °C. About 4% (V/V) seed culture was transferred to 100 ml of MSM containing various initial concentration ranging from 25 mgl^{-1} to 250 mgl^{-1} in a 250 ml Erlenmeyer flask and incubated on a rotary shaker at 27 °C. Samples were collected at different intervals of time and measured for cell growth and methylene chloride concentration. For each initial concentration of methylene chloride, specific growth rate was calculated in exponential phase. The specific growth rate (μ) in the exponential phase is calculated by the following equation: $\mu=\ln(X_2/X_1)/(t_2-t_1)$, where X_1 and X_2 are the cell dry weight obtained at time t_1 and t_2, respectively. All experiments were performed in triplicate under ideal conditions [14]. Parallel shake flask experiments are carried out to assess abiotic losses. The shake flask contained the same aqueous volume and contaminant concentration but was not inoculated. Similar set of experiment containing sterilized dead cells and methylene chloride were used to assess non-active biodegradation (i.e., binding to cell walls).

RESULTS AND DISCUSSION

1. Effect of Initial Methylene Chloride Concentration on the Culture Growth

Methylene chloride has been shown to have a significant inhibitory effect on the growth of microorganisms at higher concentrations [15]. Therefore, acclimatization of the mixed culture isolated from the municipal sewage treatment was carried out to grow in presence of methylene chloride as sole carbon source up to a maximum concentration of 250 mgl^{-1}. Initially, glucose was used during this acclimation stage, and later the culture was grown only in the presence of methylene chloride. The biomass concentration profile of mixed culture ($asOD_{600}$) at different concentration of methylene chloride is shown in Fig. 1. It observed that concentration of methylene chloride between 25 mgl^{-1} and 100 mgl^{-1} did not show any inhibitory effect on the microorganisms as indicated by almost no lag phase during its growth. However, the maximum absorbance of culture obtained using this concentration of methylene chloride was found to be less as compared, when grown in more than 100 mgl^{-1} of methylene chloride. Time taken by the culture to reach stationary growth phase of 250 mgl^{-1} was higher than the lower concentration of methylene chloride. Variation in specific growth rate with respect to initial methylene chloride concentration was observed above the concentration of 50 mgl^{-1} of methylene chloride, indicating substrate inhibition on the culture grown above the concentration as shown in Fig. 2: The specific growth rate (μ) for each initial methylene chloride concentration. Specific growth rate increased with increase in substrate concentration until 50 mgl^{-1} and then decreased with increase in substrate concentration, suggesting substrate inhibition kinetics.

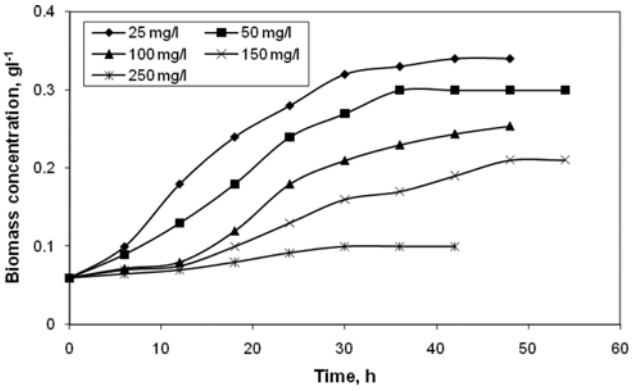

Fig. 1. Biomass growth profile for DCM biodegradation.

2. Effect of Initial Concentration on Methylene Chloride Biodegradation

Batch flask experiment was conducted to examine the effect of

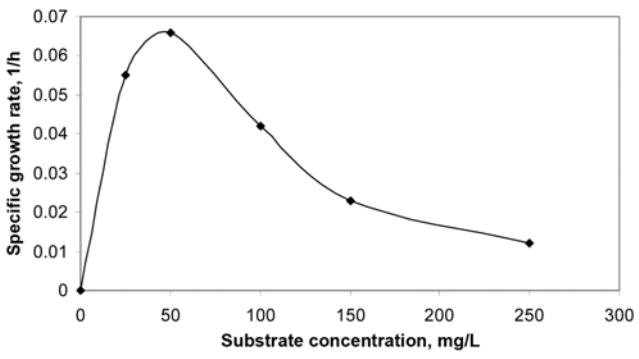

Fig. 2. Specific growth rate profile at different initial concentrations of DCM.

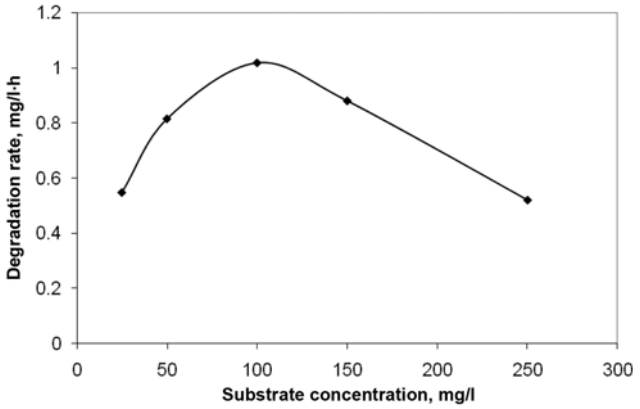

Fig. 3. Degradation rate profile of mixed cultures at different initial concentrations of DCM.

initial concentration on the degradation behavior of methylene chloride using mixed culture. Fig. 3 shows the time profile of methylene chloride degradation. The maximum degradation rate was observed at 100 mgl^{-1} of methylene chloride concentration (1 mgl^{-1} h^{-1}). Concentration below and above 100 mgl^{-1} gives lesser degradation rates by culture, which indicates the influence of methylene chloride on its degradation rate [16]. The presence of a high concentration of toxic compound like methylene chloride is reported to have an effect on growth cells, utilizing them as sole carbon and energy source, which is attributed to changes occurring at the cellular and molecular levels. The formation of acid metabolites during higher methylene chloride concentration also affects the degradation pattern. This can be observed by drop in pH from 7 to 4.5 [2]. However, the decrease in efficiency was due to toxicity toward the cells, enzyme inactivation or a combination of the two reasons [10].

Experiments were carried out for abiotic losses and non-active biodegradation (i.e., sorption to cell walls). Abiotic losses associated with volatilization were from 8% to 12%. These losses occurred within the first 10 h and were of the same magnitude for each concentration evaluated. Non–active biodegradation controls were not statistically different from their abiotic counterparts. Therefore, the primary mechanism for decrease in methylene chloride concentration was biodegradation. Similar type of result has been discussed by Shijin [17].

3. Modeling the Growth Kinetics of the Mixed Culture in Presence of Methylene Chloride

When sufficient nutrients are present, the availability of organic substrate (carbon source) is normally the rate-limiting factor. Under these conditions, the well-known, unstructured, the Monod equation is most often used to describe the relationship between the substrate concentration and the specific growth rate.

$$\mu = \mu_{max}\frac{S}{K_s + S} \tag{1}$$

However, this equation becomes unsatisfactory for explaining inhibitory growth of microorganisms at higher substrate concentration. In such case, the substrate inhibition models considered to explain the cell growth are the Haldane, Edwards and Aiba. Mathematical representations of the above model are listed below [18,19].

Model proposed by Haldane (1930):

$$\mu = \frac{\mu_{max}S}{K_S + S + \frac{S^2}{K_I}} \tag{2}$$

Model proposed by Edwards (1970):

$$\mu = \mu_{max}\left(\exp\left(-\frac{S}{K_I}\right) - \left(-\frac{S}{K_S}\right)\right) \tag{3}$$

Model proposed by Aiba et al. (1968)

$$\mu = \frac{\mu_{max}S}{K_S + S}\exp\left(-\frac{S}{K_I}\right) \tag{4}$$

where μ is the specific growth rate, 1/h; S is the initial substrate concentration, mgl^{-1}; μ_{max} is the maximum specific growth rate, 1/h; K_S is the half saturation constant, mgl^{-1}; K_I is the threshold substrate inhibition constant, mgl^{-1}.

Values of K_S indicate the ability of microbes to grow at low substrate levels [20] and K_I values indicate the sensitivity of the culture to substrate inhibition [21]. The higher K_I value physically means the culture is less sensitive to substrate inhibition and vice versa. The parameters μ_{max}, K_S and K_I from this model were evaluated by the method of least squares and implemented in the MATLAB 7.0 package. Since the models had non-linear co-efficient, the parameter

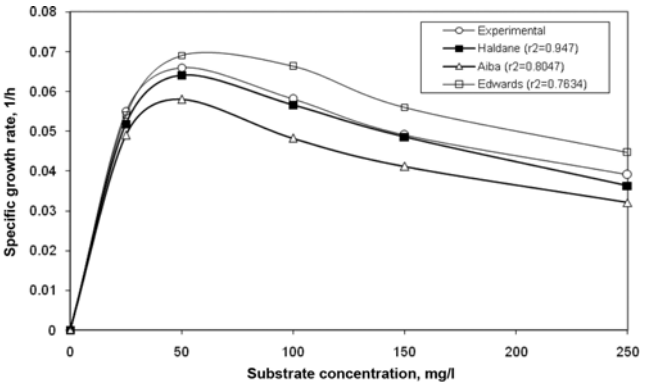

Fig. 4. Experimental and model predicted profiles of specific growth rate for DCM degradation.

Table 1. Kinetic parameters evaluated from substrate inhibition models

Models	μ_{max} (1/h)	K_S (mgl^{-1})	K_I (mgl^{-1})
Haldane	0.064	15	270
Aiba	0.058	12	255
Edwards	0.0689	28	260

Table 2. Kinetic parameters observed in literature for the biodegradation of DCM

Micro organism	μ_{max} (1/h)	K_S (mgl^{-1})	K_I (mgl^{-1})	References
Pseudomonas	0.11	17-42.5	995	Brunner et al. (1982)
Hyphomicrobium	0.11	---------	300	Diks et al. (1991)
Mixed cultures	-----	3-10	200	Niemann et al. (1993)
Mixed cultures	0.037	11.6	405	Herbst et al. (1996)

Table 3. Morphological and culture characteristics of isolated bacteria

Isolated bacteria	Gram staining	Culture characteristics on selective media
Escherichia coli	Gram negative rods	Colonies showing metallic sheen
Staphylococcus aureus	Gram positive cocci (in clusters)	Jet black colonies surrounded by white halo

were estimated iteratively with trust region nonlinear least square algorithm. Hence, μ_{max} is the maximum specific growth rate which would be obtained if the growth was not substrate inhibited and was calculated by fitting the data of uninhibited region to the Monod equation. All the models adopted in this study generally have been used to describe the substrate inhibition on growth of the microbial culture.

Fig. 4 shows the experimental specific growth rate (μ) observed at various initial methylene chloride concentration (S) which were used for estimating the kinetic parameter from the above three models. From the figure, the Haldane model was found to fit the data well. The kinetic parameters obtained from the models are shown in Table 1 along with root mean square (RMS) error between the substrate inhibition model predicted and experimental specific growth rate of the culture at various concentration. Some typical values observed from the literature on the aerobic degradation of DCM are in Table 2. It was observed that the standard deviation for the model predictions of the specific growth rate was quite low, indicating a high degree of fit for the models. However, the experimentally observed inhibitory concentrations that deviated from the model predicted inhibitory concentrations in some of the cases. Such variations in model predictions of the inhibitory concentrations are reported in the literature [20,21]. Since these models are empirical, it is not possible to use the parameter values as a measure of importance for inhibition.

4. Bacteria Isolation and Identification

Microorganism's isolation in this study was carried out from sewage of a common wastewater treatment plant. High probability of the presence of toxic pollutants in this area was the reason why the site was selected. Mixed culture was procured from common sewage waste treatment for degrading methylene chloride. Up to the three weeks, samples were enriched in sterile MSM medium using methylene chloride. After biodegradation, the bacterial strains, which survived, were identified as methylene chloride degraders. The bacterial isolates have the best potential for methylene chloride biodegradation based on high resistance of this compound. The bacterial isolate was morphologically and biochemically identified according to Bergey's manual.

Morphological and culture characteristics of isolated bacteria are listed in Table 3.

Biochemical tests were performed to confirm *E. coli* using gram staining, catalase test, indole, methyl red, Voges-Proskauer test, nitrate reduction, urease production, Simon citrate agar and various sugar fermentation tests (Table 4). Confirmation of the genus, *Staphylococcus* was done by gram staining and various biochemical tests including catalase test, oxidase test, indole, methyl red, Voges-Proskauer test, nitrate reduction, acid from different sugars, while the species, *S aureus* was confirmed by coagulase test (Table 5). The predominant microorganisms present are *Escheria coli* and *Staphylocous aureus*.

CONCLUSIONS

A mixed culture of microorganisms isolated from a common sewage treatment plant degraded methylene chloride up to a maximum concentration of 250 mgl^{-1} in mineral salt medium. The substrate inhibition due to methylene chloride and growth kinetics of culture was explained using Haldane, Aiba and Edwards substrate inhibition models. Among these models, the Haldane model gave a better fit to the experimental data. The kinetic constants evaluated using the model showed good tolerance and growth of culture and, therefore, a complete degradation of substrate. We report here bacterial strains *Escherichia coli* and *Staphylococcus aureus*, which utilize methylene chloride as sole source of carbon and energy. This work has provided a useful guideline in evaluating potential methylene chloride biodegrades isolated from the environment. In future work,

Table 4. Biochemical characterization of *Escherichia coli*

S. no	Biochemical test		Result
1	Lactose fermentation		+ve
2	Catalase		+ve
3	Simmon's (ammonium Citrate)		*−ve*
4	Indole production		+ve
5	Nitrate reduction		+ve
6	Methyl red		+ve
7	Voges-Proskauer		*−ve*
8	Urease		*−ve*
9	Acid from sugar	(a) Glucose	+ve
10		(b) Mannitol	+ve
11		(c) Lactose	+ve
12		(d) Salicin	+ve
13		(e) Sucrose	+ve

Table 5. Biochemical characterization of *Staphylococcus aureus*

S. no	Biochemical test		Result
1	Catalase		Positive
2	Oxidase		*Negative*
3	Indole production		*Negative*
4	Nitrate reduction		Positive
5	Methyl red		Positive
6	Voges-Proskauer		Positive
7	Acid from sugar	(a) Glucose	Positive
8		(b) Mannitol	Positive
9		(c) Lactose	Positive
10		(d) Raffinose	*Negative*
11		(e) Sucrose	Positive
12		(f) Maltose	Positive
13	Coagulase		Positive

this mixed culture can be utilized in bioreactors for bioremediation of methylene chloride contaminated soils, water and air.

REFERENCES

1. W. Brunner, D. Staub and T. Leisinger, *Appl. Environ. Microb.*, **40**, 950 (1980).
2. D. L. Freedman and J. M. Gossett, *Appl. Environ. Microb.*, **57**, 2847 (1991).
3. B. Herbst and U. Wiseman, *Water Res.*, **30**(5), 1069 (1996).
4. Jiade Wang and Jianmeng Chen, *Chem. Eng. J.*, **123**(3), 103 (2006).
5. Y. A. Trotsenko, N. V. Doronina, T. P. Tourova, B. B. Kuznetzov and T. Leisinger, *Systematic and Appl. Microbiol.*, **23**, 210 (2000).
6. K. D. Goodwin, J. K. Schaefer and R. S. Oremland, *Appl. Environ. Microb.*, **164**, 4629 (1998).
7. K. S. Doris and L. Thomas, *J. Bacteriol.*, **162**(2), 676 (1985).
8. K. H. Van Pee and S. Unversucht, *Chemosphere*, **52**, 299 (2003).
9. D. S. Hartmans and J. Tramper, *Bioprocess Eng.*, **6**, 83 (1991).
10. G. L. Guo, D. H. Tseng and S. L. Huang, *Biotechnol. Lett.*, **23**, 1653 (2001).
11. V. I. Krausova, F. T. Robb and J. M. Gonzalez, *J. Microbiol. Methods*, **54**, 419 (2003).
12. A. Nagamani, R. Soligala and M. Lowry, *African J. Biotechnol.*, **8**(20), 5449 (2009).
13. J. F. MacFaddin, *Biochemical tests for identification of medical bacteria*, Williams and Wilkins, London (2000).
14. S. Gokulakrishanan and N. Sathyanarayana Gummadi, *Process Biochem.*, **41**(6), 1417 (2006).
15. S. J. Wu, L. L. Zhang, J. D. Wang and J. M. Chen, *Appl. Microbiol. Biotechnol.*, **76**(6), 1289 (2007).
16. Y. Qinaruguri, N. Hiroshi, H. Wataru and O. Yukie, *Resources Processing*, **58**, 47 (2011).
17. W. U. Shijin, Y. U. Xiang, H. U. Zhihang, L. Zhang and J. Chen, *J. Environ. Sci.*, **21**, 1276 (2009).
18. Sudipta Dev and Somnath Mukherjee, *J. Water Reuse and Desalination*, **2**, 149 (2012).
19. Sudipta Dev and Somnath Mukherjee, *Int. J. Water Res. Environ. Eng.*, **2**(3), 40 (2010).
20. K. F. Reardon, D. C. Mosteller and J. D. B. Rogers, *Biotechnol. Bioeng.*, **69**(4), 387 (2000).
21. H. Shim and S. T. Yang, *J. Biotechnol.*, **67**, 99 (1999).