Steady State Characteristics of Acclimated Hydrogenotrophic Methanogens on Inorganic Substrate in Continuous Chemostat Reactors

Olga. Y. Ako[1]; Y. Kitamura[2*]; K. Intabon[2]; T. Satake[2]

[1]Doctoral Program in Bioindustrial Sciences, University of Tsukuba, Japan

[2]Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai Tsukuba Ibaraki 305-8572, Japan

*Corresponding author: Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan; tel & fax +81 029-853-4655; e-mail, kitamura@sakura.cc.tsukuba.ac.jp

Abstract

A Monod model has been used to describe the steady state characteristics of the acclimated mesophilic hydrogenotrophic methanogens in experimental chemostat reactors. The bacteria were fed with mineral salts and specific trace metals and a H_2/CO_2 supply was used as a single limited substrate. Under steady state conditions, the growth yield (Y_{CH4}) reached 11.66 g cells per mmol of H_2/CO_2 consumed. The daily cells generation average was 5.67 x 10^{11}, 5.25 x 10^{11}, 4.2 x 10^{11} and 2.1 x 10^{11} cells/ L-culture for the dilutions 0.071/d, 0.083/d, 0.1/d and 0.125/d, respectively. The maximum specific growth rate (μ_{max}) and the Monod half-saturation coefficient (K_S) were 0.15/d and 0.82 g/L, respectively. Using these results, the reactor performance was simulated. During the steady state, the simulation predicts the dependence of the H_2/CO_2 concentration (S) and the cell concentration (X) on the dilution rate. The model fitted the experimental data well and was able to yield a maximum methanogenic activity of 0.24 L CH_4/g VSS·d. The dilution rate was estimated to be 0.1/d. At the dilution rate of 0.14/d, the exponential cells washout was achieved.

Keywords: Hydrogenotrophic methanogens; chemostat; steady state; H_2/CO_2 gas; simulation; trace metals

1. Introduction

Extensive research has demonstrated the capability of methanogenic bacteria to grow on molecular hydrogen (H_2) and utilize carbon dioxide (CO_2) as an energy source (Daniels et al., 1984; Jones et al., 1985). The methanogens are inhabitants of the anaerobic fermentor, which is currently regarded as an attractive process for the degradation of various types of organic matter into methane and other by-products.

The construction of a mathematical model is obviously a crucial step in the design, control and operation of a fermentor (Bastone, 2006). Based on engineering literature, several models have been proposed to simulate and improve the fermentor performance through the supply of organic substrates (Siegrist et al., 2002; Nopharatana et al., 2003). The Monod kinetics model, which is the simplest approach, relates the specific growth rate of the biomass (μ) (1/d) to the residual substrate concentration (S) (g/L).

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

where μ_{max} is the maximum specific biomass growth rate and K_S is the half-saturation coefficient (g/L). This relationship has been used to model the single substrate H_2 consumption in the presence of another substrate (Robinson

and Tiedje, 1982). It has also been used to model anaerobic acidogenesis (Demirel and Yenigün, 2002). However, there is no report regarding the use of the Monod kinetics model for acclimated hydrogenotrophic methanogens under steady state continuous cultivation with recirculation of the single substrate H_2/CO_2. The chemostat proposed by Tang et al., 1997 was associated with steady state cultivation that provided a better conversion ratio of H_2/CO_2 to methane and carbon dioxide (Valentine et al., 2004). Four chemostat reactors were used with varying rates of flow of the inorganic medium. The objectives were, firstly, to attain a steady state chemostat cultivation of H_2/CO_2-dependant methanogens and measure the limiting supply of substrate, then to apply the Monod relationship to standardize the characteristics of H_2/CO_2 gas fermentation. The parameters obtained would provide data for the creation of a generic model for the dynamic simulation of biogas production from a methanogen culture using mixed H_2/CO_2 gas as the substrate.

2. Materials and Methods

2.1. Acclimation of hydrogenotrophic methanogens

Anaerobic activated sludge (2.5 L) obtained from a domestic waste water

treatment plant (Ibaraki, Japan) was acclimated in duplicate 5 L reactors at mesophilic temperatures for 7 months; H_2/CO_2 (80:20, v/v) gas was used as source of energy and carbon. Using a 2 L gas bag, the gas substrate was recirculated at 0.08 MPa. The archaea cultivation procedure (Sowers and Noll, 1995) was implemented and continued until H_2/CO_2-dependant methanogens predominated the culture. The physical and chemical compositions of the acclimated sludge obtained were averaged from the 2 reactors and are shown in Table 1. According to these results, the acetic acid concentration was nil and 56.6% methane was produced. The absence of acetic acid indicates the possibility that methane production from substrates other than the H_2/CO_2 gas mixture can be excluded.

2.2. Epifluorescent microscopy analysis

The acclimated sludge samples were filtered using a polycarbonate black filter with 0.22 μm pore diameter. Using ethidine bromide (Sigma-Aldrich, Poland) as the stain, the sludge samples were observed under an ultra-microscope (OLYMPUS, BX 50, Japan). The apparently uniform blue autofluorescence verified the purity of the culture. A morphological examination of the bacteria revealed long rod-shaped cells and cocci.

2.3. Experimental procedure for continuous chemostat reactors

A modified version of the medium used by Yang et al. (2004) was applied; the final composition was as follows: mineral nutrients (in g/L of demineralized water) including K_2HPO_4, 3.4; KH_2PO_4, 3.4; Na_2CO_3, 2.54; NH_4Cl, 2.13; and trace metals (in mg/L of demineralized water) including $MgSO_4 \cdot 7H_2O$, 600; $MnSO_4 \cdot 5H_2O$, 5.3; $NiCl_2 \cdot 6H_2O$, 125; $FeSO_4 \cdot 7H_2O$, 28; $CoCl_2 \cdot 6H_2O$, 10; $ZnSO_4 \cdot 7H_2O$, 30; $CuSO_4 \cdot 5H_2O$, 8; $AlK(SO_4)_2 \cdot 12H_2O$, 1; $CaCl_2 \cdot 2H_2O$, 40; H_3BO_4, 1 and $Na_2MoO_4 \cdot 2H_2O$, 1.5. The mineral nutrients and trace metals were prepared anaerobically and the estimated pH values were 7.95 and 6.93, respectively. The media were stocked in two 5 L Duran vials. In order to maintain an anaerobic environment, the headspaces were flushed with H_2/CO_2 (80:20, v/v) for 15 min at 0.1 MPa.

Chemostat cultivation: Using different dilution rates (D), i.e. $D_1 = 0.071/d$, $D_2 = 0.083/d$, $D_3 = 0.1/d$ and $D_4 = 0.125/d$ of the nutrients and fixed amounts of the trace metals (5 mL), the acclimated sludge was grown in 4 chemostats comprising semi-continuously stirred tank reactors of 1 L, each with a 500 mL working volume incubated at 37 ± 2ºC. The chemostat reactors were connected to four airtight pumps for the recirculation of H_2/CO_2 (80:20, v/v). Each pump was

connected to a timer working at 1 min intervals; the flow rate of H_2/CO_2 (80:20, v/v) gas was maintained at 0.83 mL/min at 0.08 MPa to maximize the mass transfer between the gas and the liquid (Coates et al., 1996). The liquid growth culture was taken from the reactors and replaced anaerobically with inorganic nutrients and trace metals at the dilution rates stated above. A new aluminum GL Sciences gas sampling bag (CCK) consisting of two mini valves containing pure H_2/CO_2 gas was connected to the recirculation gas system at 24 h intervals to obtain a daily estimate of the CH_4 gas produced during the experiment until a the steady state fermentation was achieved.

2.4. Analytical methods

The composition of the gas produced was determined using a GC-14B (Shimadzu) gas chromatograph equipped with a thermal conductivity detector connected to a C-R8A data analyzer. A high performance column packed with 50/80 mesh Porapak Q was used. The temperatures of the injection, column and detector were set to 100, 50, 100ºC, respectively. Argon was used as the carrier gas at a pressure of 0.5 MPa. The total solids (TS), volatile suspended solids (VSS) and volatile solids (VS) were determined with 5 duplicate analyses to ensure accuracy of the results obtained, according to the Standard Methods for

Examination of Water and Wastewater (APHA, 2005). The data were subsequently averaged; the deviations obtained were found to be less than 4%. The pH was monitored *in situ*. Cell number was estimated by a direct count using fluorescence microscopy (OLYMPUS, BX 50).

3. Results and Discussion

3.1. Steady state characteristics of chemostat H_2/CO_2-dependant methanogens

Table 2 shows the measurements of the decrease in the gas concentration from the replaced aluminum gas sampling CCK bag. In the steady state, the percentage consumption rates of H_2 and CO_2 estimated individually, are approximately equal, and the equilibrium ratio did not change with variations in the dilution rates. The results suggest that the H_2/CO_2 complex gas mixture can be considered as a single substrate during the determination of the kinetics parameters of these bacteria. The pH was not found to be a significant factor in the inhibition of bacterial activity within the continuously flowing reactor (Keshtkar et al., 2003) for any dilutions; this is due to the buffering effect of the media and the dissolved H_2/CO_2 in the liquid. However, the inhibitory effect of pH on the physiological activities of hydrogenotrophic methanogens varies among different species

(Savant et al., 2002; Kotsyurbenko et al., 2004; Wolfe and Higgins, 1979). Costello et al. (1991) presumed that the Monod kinetics can be regarded as a mechanism for correction of the pH inhibition during the determination of the kinetic parameters. As presented in Fig.1, cell production under these conditions illustrates that the biomass concentration decreases with an increase of dilution, data similar to the results obtained by Haydock et al. (2004). The daily cells generated measurements were 5.67×10^{11}, 5.25×10^{11}, 4.2×10^{11} and 2.1×10^{11} cells/L-culture for 0.073/d, 0.083/d, 0.1/d and 0.125/d, respectively. No bacteria carrier was used in the different reactors; thus, the dilution effect explains the data. The specific methanogenic activity (SMA) at different dilutions was determined according to the Ahring and Sorensen (1993) and the results are presented in Fig. 2. The specific methane production rate was varied from 0.6 to 0.7 mmol/g cell hr, values lower than the 7.5 to 11.3 mmol/g cell hr determined by Wise et al. (1978) under the same conditions for a cell recycling system. In the steady state, the highest hydrogenotrophic methanogen activity was observed at a dilution of 0.1/d, 0.24 L CH_4/g VSS·d, due to the low quantities of trace metals available at low dilution rates. The methanogenic activity plot suggests a decoupling mechanism of anabolism and catabolism with regard to the metabolic activity of

hydrogenotrophic methanogens (Schönheit et al., 1980; Fardeau et al., 1987).

3.2. Kinetic evaluation in chemostat reactor using H_2/CO_2 as substrate

In chemostat cultivation, the Monod equation is expressed as follows:

$$\frac{1}{D} = \frac{K_S}{\mu_{\max}}\frac{1}{S} + \frac{1}{\mu_{\max}} \quad (2)$$

where D is the dilution rate (1/d).

Under steady state conditions, the bacterial growth yield ($Y_{X/S}$) is expressed as follows:

$$Y_{X/S} = \frac{X}{S_0 - S} \quad (3)$$

where S_0 and S represent the concentration of substrate (g/L) at the start of the experiment and at time t, respectively. X represents the biomass concentration (g/L).

Considering the complex gas mixture of H_2/CO_2 as a single substrate, K_s and μ_{max} were determined from Eq. (2) (Lee, 1992). The experimental results from the chemostat cultivation showed a maximum growth yield of 11.66 g cells

for each mmol of H_2/CO_2 substrate consumption. This growth rate was found to be higher than the ranges of 0.0053~0.0095 g/mmol and 0.00279~0.0079 g/mmol that were obtained using a pure mixture of *Methanosarcina* sp. strain 227 and *Methanosarcina mazei* under H_2/CO_2 in media containing minerals (Ferguson and Mah, 1983). The operational design of our experiment reveals a good productive fermentation system. The saturation constant K_S was 0.82 g/L, greater than the 0.116 g/L that is necessary for methanogenic bacteria when they compete with sulphate-reducing bacteria for acetate (Bhattacharya et al., 1996) or other substrates (Gilardo-Gomez et al., 1992). With inorganic materials as substrate, it is suggested that a high concentration of H_2/CO_2 is necessary to achieve the cells production. However, the maximum specific growth obtained with H_2/CO_2 as a unique substrate was 0.15/d, and it is 10 times lower than the concentration 1.5/d that was reported for H_2 and CO_2 when acclimated mesophilic methanogen culture was grown using separate organic substrates for H_2 and CO_2 (Zhang and Maekawa, 1994). The flow rate of the gas mixture applied in the present study was 8.3 mL/min, a value substantially lower than that applied by Zhang & Maekawa (1994). The results presented here correspond well with those of

previous literature regarding the maximum specific growth rate as a function of gas flow rate (Schill et al., 1996).

3.3. Simulation

To simulate the behavior of the substrate (*S*), the cell production rate (*DX*) and the cell concentration (*X*) in the chemostat reactor under steady state conditions, the following relations are used:

$$S = \frac{K_S D}{\mu_{\max} - D} \tag{5}$$

$$X = Y_{X/S}\left(S_0 - \frac{K_S D}{\mu_{\max} - D}\right) \tag{6}$$

$$DX = Y_{X/S} D\left(S_0 - \frac{K_S D}{\mu_{\max} - D}\right) \tag{7}$$

The washout occurs when the cell concentration is equal to zero

$$D_{crit} = \mu_{\max} \frac{S_0}{K_S + S_0} \tag{10}$$

The slow growth of the CO_2-reducing methanogens normally involve a retention time greater than 10 d for efficient and stable operation (Zinder, 1993). Fig. 3 summarizes the predictive plots for the hydrogenotrophic methanogen

growth and substrate supply at different dilution rates. The graphs indicate the maximum bacterial rate of growth at low dilution. This amount decreases as the dilution increases until it reaches D_m, the maximum dilution where the amount of bacteria in the reactor due to the dilution rate is in equilibrium with the limited supply of nutrients and trace metals. Subsequently, the cell washout begins to reach D_{crit} at the high dilution rate of 0.14/d; the predicted D_m was estimated as 0.11/d. However, the cell production rate (DX) increases with increasing dilution rate. The experimental data were in agreement with the predictive plots. The proposed operation system shows that the rates of dilution must be around 0.11/d to attempt a short retention time and a high substrate conversion rate at relatively low cell concentration in order to obviate the problems due to long retention time and toxicity effects of the trace elements. In the present study, the methanogenic bacteria attain an efficient activity level after a short retention time. This prediction was verified by the SMA previously determined (Fig. 2) where the highest methanogenic bacterial activity was 0.24 L CH_4 /g VSS·d, detected at 0.1/d; this value was found to be close to the predicted value of D_m, which was 0.11/d. The predicted critical dilution (D_{crit}) value was 0.14/d. The CH_4 production effectively dropped when the dilution increased beyond D_{crit}. Table 3

presents a suitable range of parameters for chemostat cultivation with a mixture of H_2/CO_2 (80:20, v/v) as substrate which can serve to design a reactor plant.

4. Conclusion

Four anaerobic continuously stirred tank reactors containing cultures of acclimated hydrogenotrophic methanogen, obtained from a domestic anaerobic wastewater treatment plant, were run at different dilution rates in order to obtain engineering data necessary to simulate steady state chemostat cultivation with a supply of inorganic substrate. Under the conditions of steady state cultivation, the Monod model was applied to determine the kinetic parameters, μ_{max} and K_S. The values found were 0.15/d and 0.82 g/L, respectively. From these results, a simulation was performed to predict the dilution rates at which bacteria washout began and at which maximum bacterial activity could be achieved for a concentration of 11.14 g/L of H_2/CO_2. The critical dilution rate for the chemostat was 0.14/d, and the maximum bacterial activity occurred at 0.11/d. The predictions agreed with the data obtained; in addition, the maximum methane gas production rate of 1.21 L/d was achieved at a dilution of 0.1/d. Thus, the engineering results can be summarized and applied to the large scale use of

H_2/CO_2-dependant methanogens for biogas production using only inorganic substrates. The results demonstrated that, at steady state, the concentration of cells was related more closely to the dilution rate than the H_2/CO_2 concentration.

Acknowledgements

This research was partially supported by the Ministry of Education, Science, Sports and Culture, Grant-in-Aid for Scientific Research, 14560216, 2003.

References

Ahring, B.K., Sorensen, H.A., 1993. Measurements of the specific methanogenic activity of anaerobic digestor biomass. Appl. Microbiol. Biotechnol. 40, 427-431.

APHA-AWWA-WEF, 2005. Standard Methods for the Examination of Water and Wastewater, 21st ed., American Public Health Association, Washington, DC.

Batstone, D.J., 2006. Mathematical modelling of anaerobic reactors treating

domestic wastewater: Rational criteria for model use. Rev. Environ. Sci. Biotechnol. 5, 57–71.

Bhattacharya, S.K., Uberoi, V., Dronamraju, M.M., 1996. Interaction between acetate fed sulfate reducers and Methanogens. Water Res. 30, 2239-2246.

Coates, J.D., Coughlan, F.M., Colleran, E., 1996. Simple method for the measurement of the hydrogenotrophic methanogenic activity of anaerobic sludges. J. Microbiol. Methods 26, 237-246.

Costello, D.J., Greenfield, P.F., Lee, P.L., 1991. Dynamic modelling of a single-stage high-rate anaerobic reactor-II. Model verification. Water Res. 25, 859-871.

Daniels, L., Sparling, R., Sprott, G.D., 1984. The bioenergetics of Methanogenesis. Biochim. Biophys. Acta 768, 113-163.

Demirel, B., Yenigün, O., 2002. Two-phase anaerobic digestion processes: a review. J. Chem. Technol. Biotechnol. 77, 743-755.

Fardeau, M-L., Peillex, J-P., Belaïch, J-P., 1987. Energetics of the growth of *Methanobacteriuum thermoautotrophicum* and *Methanococcus thermolithotrophicus* on ammonia chloride and dinitrogen. Arch. Microbiol. 148, 128-131.

Ferguson T.J., Mah, R.A., 1983. Effect of H_2-CO_2 on Methanogenesis from Acetate or Methanol in *Methanosarcina* spp. Appl. Environ. Microbiol. 46, 348-355.

Giraldo-Gomez, E., Goodwin, S., Switzenbaum, M.S., 1992. Influence of mass transfer limitations on determination of the half saturation constant for hydrogen uptake in a mixed-culture CH_4-producing enrichment. Biotechnol. Bioeng. 40, 768-776.

Haydock, A.K., Porat, I., Whitman, W.B., Leigh, J.A., 2004. Continuous culture of *Methanococcus maripaludis* under defined nutrient conditions. FEMS Microbiol. Lett. 238, 85-91.

Jones, W.J., Donnelly, M.I., Wolfe, R.S., 1985. Evidence of a Common Pathway of Carbon Dioxide Reduction to Methane in Methanogens. J. Bacteriol. 163, 126-131.

Keshtkar, A., Meyssami, B., Abolhamd, G., Ghaforian, H., Khalagi, M.A., 2003. Mathematical modeling of non-ideal mixing continuous flow reactors for anaerobic digestion of cattle manure. Bioresour. Technol. 87, 113-124.

Kotsyurbenko, O.R., Chin, K.J., Glagolev, M.V., Stubner, S., Simankova, M.V., Nozhevnikova, A.N., Conrad, R., 2004. Acetoclastic and hydrogenotrophic

methane production and methanogenic populations in acidic West-Siberian peat bog. Environ. Microbiol. 6, 1159-1173.

Kun, L.Y., 2003. Bioprocess technology, in: Kun, L.Y. (Ed.), Microbial Biotechnology Principles and Applications. World Scientific Publishing, Singapore, pp. 23-68.

Lee, J.M., 1992. Cell Kinetics and Fermenter Design, in: Lee, J.M. (Ed.), Biochemical engineering. Prentice-Hall, New Jersey, pp. 138- 163.

Nopharatana, A., Pullammanappallil, P.C., Clarke, W.P., 2003. A dynamic mathematical model for sequential leach bed anaerobic digestion of organic fraction of municipal solid waste. Biochem. Eng. J. 13, 21-33.

Robinson, J.A., Tiedje, M.J., 1982. Kinetics of hydrogen Consumption by Rumen Fluid, Anaerobic Digestor Sludge and Sediment. Appl. Environ. Microbiol. 44, 1374-1384.

Savant, D.V., Shouche, Y.S., Prakash, S., Ranade, D.R., 2002. *Methanobrevibacter acididurans* sp.nov., a novel methanogen from a sour anaerobic digester. Int. J. Syst. Evol. Microbiol. 52, 1081-1087.

Schill, N., van Gulik, M.W., Voisard, D., von Stockar, U., 1996. Continuous cultures limited by a gaseous substrate: Development of a simple,

unstructured mathematical model and experimental verification with *Methanobacterium thermoautotrophicum*. Biotechnol. Bioeng. 51, 645-658.

Schönheit, P., Moll, J., Thauer, R.K., 1980. Growth parameters (K_S, μ_{max}, Y_S) of *Methanobacterium thermoautotrophicum*. Arch. Microbiol. 127, 59-65.

Siegrist, H., Vogt, D., Garcia-Heras, J.L., Gujer, W., 2002. Mathematical model for meso- and thermophilic anaerobic sewage sludge digestion. Environ. Sci. Technol. 36, 1113-1123.

Sowers, K.R., Noll, K.M., 1995. Techniques for anaerobic growth. in Sowers K.R., Schreier H.J. (Eds.), Archaea A laboratory manual Methanogens. Cold Spring Harbor Laboratory Press, USA, pp. 15-46.

Tang, B., Sitomer, A., Jackson, T., 1997. Population dynamics and competition in chemostat models with adaptive nutrient uptake. J. Math. Biol. 35, 453-479.

Valentine, D.L., Chidthaisong, A., Rice, A. Reeburgh, W.S., Tyler, S.C., 2004. Carbon and hydrogen isotope fractionation by moderately thermophilic methanogens. Geochim. Cosmochim. Acta 68, 1571-1590.

Wise, D.L., Cooney, C.L., Augenstein, D.C., 1978. Biomethanation: Anaerobic fermentation of CO_2, H_2 and CO to methane. Biotechnol. Bioeng. 20, 1153-1172.

Wolfe, R.S., Higgins, I.J., 1979. Microbial biochemistry of methane- a study in contrasts. Int. Rev. Biochem. 21, 267-353.

Yang, Y., Zhang, Z., Lu, J., Maekawa, T., 2004. Continuous methane fermentation and the production of vitamin B_{12} in fixed- bed reactor packed with loofah. Bioresour. Technol. 92, 285-290.

Zhang, Z.Y., Maekawa, T., 1994. CH_4 fermentation using Acclimated Methanogens on a continuous feed substrate of carbon dioxide and hydrogen. Journal of the Society of Agricultural Structures. Japan, 24 (4), 207-214.

Zinder, S.H., 1993. Physiological Ecology of Methanogens. in: Ferry, J.G. (Ed.), Methanogens Ecology, Physiology, Biochemistry & Genetics. Chapman & Hall Microbiology series, New York, pp. 128-206.