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CO Fermentation of *Eubacterium limosum* KIST612

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Abstract *Eubacterium limosum* KIST612 was cultured on phosphate-buffered basal medium (PBBM) with carbon monoxide (CO) as the sole energy and carbon source. The initial growth rate of this strain was approximately 0.17~0.25 h^{-1} and the K_s value for dissolved substrate was 0.14 mM. CO was limiting during the growth of the bacterium when the CO partial pressure was less than 0.6 atm (0.5 mM dissolved CO). The bacterial growth rate was reduced in the presence of acetate. When sufficient CO was supplied using a gas-lift reactor, the acetate concentration went up to 90 mM in 116 h. Based on these findings, it is suggested that a pressurized reactor be used to develop a process to convert CO-rich gases into multi-carbon compounds.

Key words: Acetogen, *Eubacterium limosum*, CO utilizing anaerobe, acetate, organic acid production

The low cost of coal reserves and the availability of various coal processing technologies have engendered a great deal of interest in the developments of coal based alternative energy production routes [7, 31, 35]. Synthesis gas (syn-gas) produced from the gasification of not only coal but also biomass will be a prime alternative feedstock for petroleum. The main component of syn-gas is carbon monoxide (CO) which can be converted to multi-carbon compounds for the purpose of chemicals production [9, 27]. Like the syn-gas, off-gases from steel industries contain a high concentration of CO [6]. The off-gases are burnt to produce large quantities of carbon dioxide, a green-house effect gas.

An efficient process converting CO to multi-carbon compounds can reduce CO_2 emission from the steel industry with the production of chemical feedstock which can substitute for those derived from the petrochemical industry.

CO can be converted to multi-carbon compounds by catalytic processes such as the Fisher Tropsch process. These processes are very expensive to operate. In addition to the higher energy requirement for high operating temperatures and pressures, CO should be purified and concentrated because the sulfur compounds in gases poison the catalysts [1, 2, 32]. The bioconversion of syn-gas produced from the gasification of coal or biomass will also improve the utilization and economics of multi-carbon chemical synthesis. A group of anaerobic bacteria, the homoacetogens, can convert CO to multi-carbon compounds such as acetic and butyric acids, and they have been used to develop a biological CO conversion process [7, 13, 15, 35]. These bacteria are resistant to the sulfur compounds in the gases, which can stimulate the growth of the anaerobic bacteria as they remove molecular oxygen (O_2) from the growth environment.

Homoacetogens grow chemolithotrophically under anaerobic conditions, reducing CO_2 as the terminal electron-acceptor to acetyl-CoA through an energy-conserving process [10, 11, 18]. Unlike the classical two-stage vinegar process where hexose is subjected to yeast fermentation to ethanol, which is then oxidized to acetate by acetic acid bacteria, homoacetogens ferment one mole of hexose to three moles of acetate directly [11, 18], conserving all carbons of the substrate in the product. Like most of the organic acid fermentation processes, the acetogenesis is inhibited by its product, acetate, even more at low pH [20, 30, 33, 34]. This has a significant influence on process productivity with low final product concentration and on product formation kinetics.

The growth of a variety of acetogens has been studied and growth rates are known to be significantly affected

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by CO concentration. A mutant of *Butyribacterium methylotrophicum* Marburg strain was adapted to grow with a 12 h doubling time on 100% CO under 1 atm (101.3 kPa) [23]. A sheep rumen isolate, *Eubacterium limosum* RF strain, produced acetate with a 7 h doubling time when grown on 50% CO (total pressure, 202.6 kPa; $N_2/CO_2/CO$, 30/20/50). However, the doubling time increased to 18 h in a 75% CO gas phase [14]. *Peptostreptococcus productus* U-1, isolated from anaerobic sewage digester sludge, has a minimum doubling time of 1.5 h in 50% CO (total pressure, 202.6 kPa; $N_2/CO_2/CO$, 30/20/50). However, the log phase was prolonged with over 80 kPa of CO partial pressure [12]. The thermophile *Clostridium thermoaceticum* exhibited a doubling time of 18 h when grown on CO [16].

Acid tolerant strains have been obtained from *Clostridium thermoaceticum* [3, 26] and *Acetogenium kivui* [12, 17]. In a fed-batch process controlled at pH 6.2, the mutant of *C. thermoaceticum* produced 52.5 g/l acetic acid in 140 h, and the parent strain produced 44 g/l in 192 h [26]. In pH-controlled batch fermentation at pH 6.4, *A. kivui* was able to produce up to 625 mM (37.5 g/*l*) of acetic acid from glucose in 50~60 h [17]. This strain also showed 700 mM of acetic acid production in 40~50 h in fed-batch fermentation. According to these studies, it is postulated that production costs of the acetogenic process could outcompete synthetic processes if an acid tolerant, high acetic acid-producing acetogen were to be obtained.

Acetic acid is an important organic compound in the petrochemical industry. It is produced synthetically from fossil feedstocks such as methanol. In 1989, approximated 8 billion lbs of acetic acid were produced worldwide with half of it coming from the US [4, 11]. The oil crises fueled interest in the commercial application of acetogenesis for the production of acetic acid. However, such interest yielded mainly laboratory-level investigations rather than process-level applications because prices and availability of oil returned to levels that did not necessitate a shift away from synthetic processes. Nonetheless, acetic acid production from a renewable resource seems inevitable due to the limited reserve of petroleum and to the green house effects of fossil fuel use [11].

Previous studies have shown that biotechnological processes can be economically feasible should an acid tolerant, high acetic acid-producing acetogen be obtained [3, 12, 20, 30]. The present study was conducted to evaluate *E. limosum* KIST612 as a means of converting CO rich gases such as off-gas from steel mills to multi-carbon compounds in terms of acid tolerance of the bacterium. Also studied was the mass transfer and limitation of the gaseous substrate. Few studies have been reported on the subject.

MATERIALS AND METHODS

Bacterial Strain and Its Cultivation

E. limosum KIST612 [6] was used throughout the study. Phosphate buffered basal medium (PBBM) was used to cultivate and maintain the culture [5]. The medium was prepared under strictly anaerobic conditions with N_2 headspace using pressure tubes (Bellco Glass Inc., Vineland, NJ, U.S.A.) or serum vials (Wheaton Scientific Co. Milliville, NJ, U.S.A.). The head space of the culture vessel was pressurized to 2 atm using pure CO before autoclaving at 121°C for 20 min. The cultures were initiated with a 5% (v/v) inoculum of fresh culture.

Batch cultures were made using serum vials in a shaking water bath (150 rpm, JeioTech, Seoul, Korea) at 37°C without pH control. A gas-lift type reactor was also used with a working volume of 0.2 litre. The pH was automatically controlled by the addition of a 2 N NaOH solution. The temperature was maintained at 37°C. To avoid the oxidation of the system due to the negative pressure caused by the consumption of CO, a gas-tight bag with a volume of 1.45 or 11.8 litres (Alltech, Deerfield, IL, U.S.A.) was connected to the reactor as shown in Fig. 1. CO was supplied to the reactor as the sole energy and carbon source by sparging continuously at a rate of 80 ml/min through a sintered glass (60/80 mesh).

Analyses

Cell concentration was determined by measuring the optical density at 660 nm using a spectrophotometer (Jasco UVIDEC-610, Tokyo, Japan). Cell mass was calculated using a previously-derived dry weight versus optical density calibration curve. An $OD_{660}^{1\,cm}$ of 1.0 corresponded to 0.271 g of cells per litre for CO-grown cells.

Volatile fatty acids were quantified with a gas chromatograph as described previously [6]. The CO and CO_2 were measured by a thermal conductivity detector with a gas chromatograph as described previously [6]

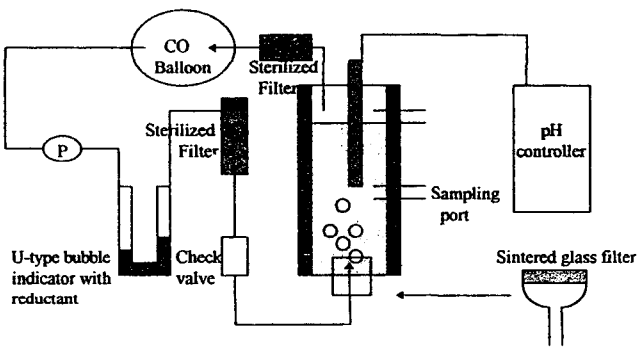

Fig. 1. Schematic diagram of an acetogenic reactor using CO as substrate.

using a gas-tight syringe (A-2 Gas Pressure-Lok, Dynatech, Baton Rounge, LA, U.S.A.).

Dissolved CO and CO_2 were measured after the gases were transferred to gas phase by acidification and heating. Two ml of culture were transferred to a pressure tube containing 1 ml of 1 N HCl before being heated at 105°C in a heating block (MULTI-BLOCK Heater, LAB-LINE Instrument Inc., Meirose, IL, U.S.A.) for 10 min. Standard gases were supplied by Alltech (CTR I Calibration Gas, Scotty I Analyzed Gases).

RESULTS

Stoichiometry of CO Fermentation by *E. limosum* KIST612

Cultures were made using serum vials and incubated for 72 h before analyses were made to determine the CO fermentation balance (Table 1). Since yeast extract was used in the medium, the calculations were made from the stoichiometric differences between cultures with and without CO. The fermentation stoichiometry was found to be 3.312 CO → 1.98 CO_2 + 0.573 acetate + 0.05 butyrate. The carbon recovery was 98.9%, and that of electron was 84.2%. Trace amounts of hydrogen were determined after the fermentation, but were not considered in the calculations of the fermentation stoichiometry and electron recovery. It was assumed that carbon and electron not recovered in the products were used to synthesize cell mass.

Effect of CO Concentration on the Growth of *E. limosum* KIST612

CO is a substrate for homoacetogens and, at high concentration, inhibits bacterial growth. Growth rates of *E. limosum* KIST612 were determined using pressure tubes containing different amounts of CO in the head space. In order to determine the growth in terms of dissolved CO, dissolved gas was determined as described in the Materials and Methods after the tubes were equilibrated at the growth temperature (Fig. 2). CO solubility was approximately 0.8 mmol/l of PBBM under 101.3 kPa of CO partial pressure at 37°C. This value is similar to CO solubility of 0.84 mmol/l water at 35°C [36].

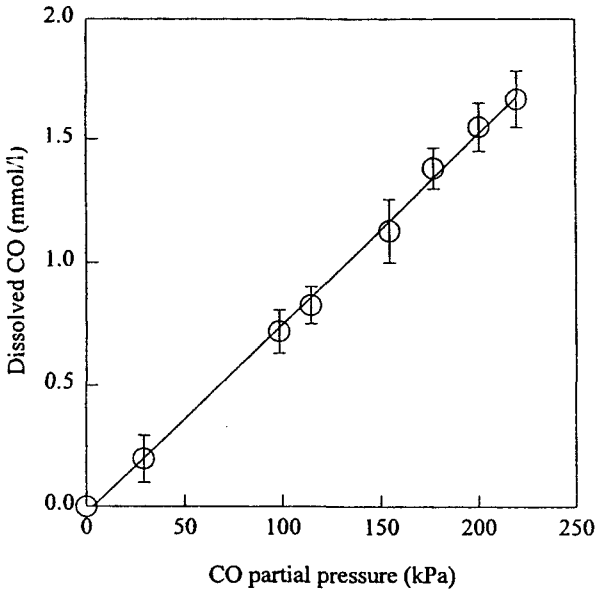

Fig. 2. Relationship of CO partial pressure and CO solubility in PBBM.

CO was added to anaerobic pressure tubes to obtain the desired CO partial pressure with N_2 balance before samples were taken to measure dissolved gas by acidification and heating. Results are mean values of triplicate determinations.

Table 1. CO fermentation balance by *E. limosum* KIST612.

Medium	CO	CO_2	Acetate	Iso-butyrate	*n*-butyrate
PBBM with CO					(mmol)
Initial	3.320	0.05	0.068	-	-
Final	0.008	2.09	0.710	0.045	0.105
Net	3.312	2.04	0.642	0.045	0.105
PBBM* without CO					
Initial	-	-	0.043	-	-
Final	-	0.06	0.112	0.029	0.071
Net	0	0.06	0.069	0.029	0.071
Net balance	3.312	1.98	0.573	0.016	0.034
Carbon and electron recoveries, and O/R balance					
Carbon recovery					98.9%
Electron recovery					84.2%
O/R balance	substrate				-0.53
	products				

The substrate-product stoichiometry was calculated as: 3.312 CO → 1.98 CO_2 + 0.573 CH_3COOH + 0.05 $CH_3CH_2CH_2COOH$.

* Phosphate-buffered basal medium.

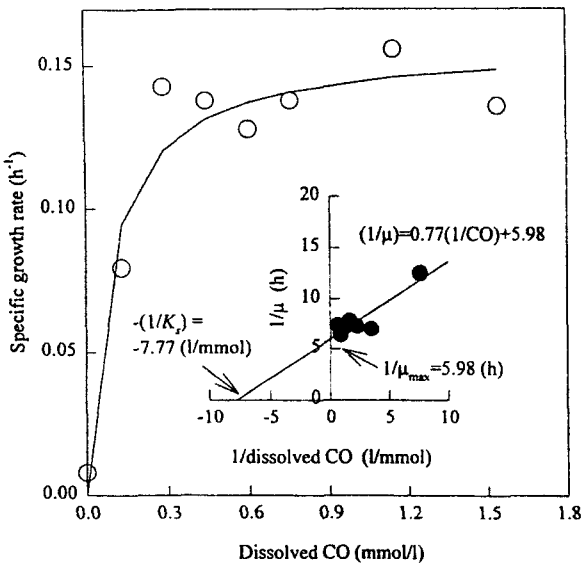

Fig. 3. Relationship between CO partial pressure and specific growth rate of *E. limosum* KIST612.

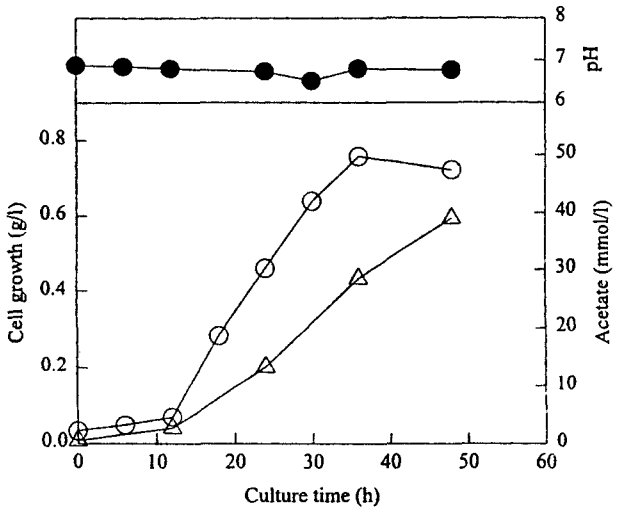

Fig. 4. The growth of *E. limosum* KIST612 on PBBM with CO.

The gas-lift reactor (Fig. 1) was used with 100% CO. The culture was initiated by 5% (v/v) inoculation. pH (●) was controlled to 6.8 by addition of 2 N NaOH. Optical density (○) and acetate concentration (△) was determined every 6 h, and CO consumption was determined at the end of culture.

Figure 3 shows the relationship between the dissolved CO concentration and growth rate of *E. limosum* KIST 612. The apparent K_s value was about 0.14 mM CO, and the maximum growth rate about 0.17 h^{-1}. The growth was not inhibited at dissolved CO concentrations up to 1.5 mM which was equivalent to 1.8 atm of CO.

pH Controlled CO Fermentation

The bacterium was grown using the gas-lift reactor with CO for 2 days with pH control to reduce the inhibitory effect of acidic fermentation products. Bacterial growth and formed acetate were measured every 6 h, and CO consumption and CO_2 production were measured at the end of the fermentation. The specific growth rate was measured to be 0.232 h^{-1} (mass doubling time: 3 h) at initial log phase, and decreased gradually with the accumulation of acetate (Fig. 4). At the end of the culture, the cell mass decreased slightly with an acetate concentration of 30 mM, which is equivalent to 7.9 mmol of acetate. Acetate production continued after the culture stopped growing at 36 h. These results show that the maintenance energy requirement is higher than the energy available from CO conversion to acetate at this condition. Besides acetate, trace amounts of butyrate and ethanol were detected as minor soluble products at the end of the fermentation.

The volume of the gas-tight gas bag decreased from 1.45 to 0.65 liters with a CO/CO_2 ratio of 6.0/3.5. The total volume of the gas phase is 0.85 liters with 0.2 litre headspace of the reactor. The CO consumption was calculated to be 1.065 liter (43 mmol), and the cell mass produced was 0.15 g. The total amount of CO_2 produced was measured to be 19.9 mmol–11.8 mmol in the gas phase and 8.1 mmol dissolved in the medium. The overall stoichiometry was calculated as; 43 CO → 19.9 CO_2 + 7.9 acetate + 0.6 butyrate + 0.8 ethanol with carbon and electron recoveries of 92.3 and 98.6%, respectively. In this experiment, the acetate yield was slightly higher (0.18 acetate/CO) than that of the vial culture (0.17 acetate/CO). However, the butyrate yield (0.014 butyrate/CO) was lower than that of the vial culture (0.015 butyrate/CO). The differences seem to be due to the pH control.

Effect of Added Acetate on the Growth of *E. limosum* KIST612 with CO

The specific growth rate decreased as the fermentation proceeded with the accumulation of acetate (Fig. 4). PBBM with 1 atm (101.3 kPa) of CO was added with sodium acetate up to 240 mM to study the effects of acetate on the growth of the bacterium. As shown in Fig. 5, the specific growth rate decreased as the acetate concentration increased, and the pH changes were less significant with the added acetate. Acetate did not affect bacterial growth at low concentrations up to 40 mM, and the bacterium grew in the presence of acetate up to 240 mM. The decrease in the growth rate was not as significant as that in the batch culture [5]. This might have been due to the higher buffering capacities of the media by the addition of sodium acetate.

Effect of CO Refreshment on the Growth of *E. limosum* KIST612

As shown in Fig. 4, the bacterial growth ceased when the acetate concentration reached around 39 mM

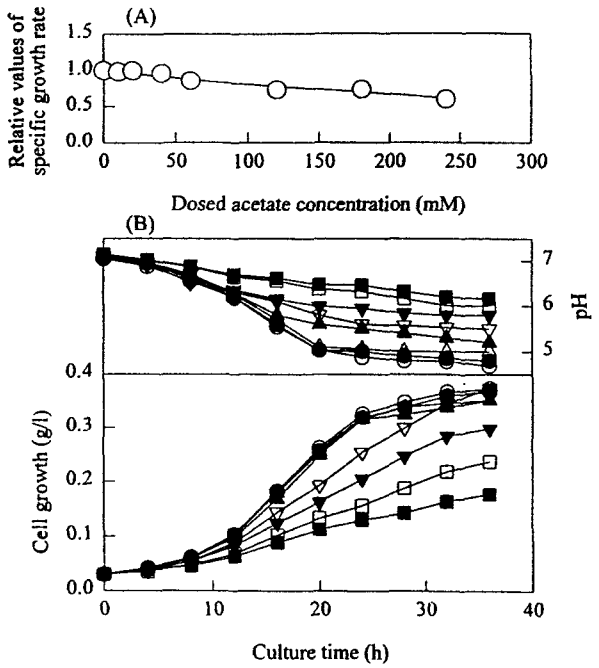

Fig. 5. The effect of acetate concentration on the growth of *E. limosum* KIST612.

The acetate concentrations added to the culture with 1 atm CO partial pressure were: 0 (○), 10 (●), 20 (△), 40 (▲), 60 (▽), 120 (▼), 180 (□), and 240 (■) mM. Results are mean values of duplicates. (A): relative values of specific growth rate, (B): growth profile and pH. Relative value of specific growth rate was calculated by the following equation; Relative values=(specific growth rate of treatment/specific growth rate of control).

with a residual CO partial pressure of 0.6 atm. However, the bacterium could grow in the presence of acetate up to 240 mM (Fig. 5). Studies were done using the gas-lift reactor to see if the CO supply is the limiting factor for the cell growth. The reactor was run as in Fig. 4, and the gas-tight bag was changed to supply fresh 100% CO at 24 and 48 h after the initiation of growth (Fig. 6). After the first change of fresh CO, the bacterial growth continued beyond 1.0 g/l, which is higher than the maximum cell concentration without CO refreshment. The bacterial growth after the second change was much slower than the first one. The slow growth in Fig. 6 seems due to the fact that cells in high concentration consume CO at a higher rate than CO transfer into liquid. These results show that CO transfer is limiting. The cease of growth in Fig. 4 seems due to slow CO transfer to meet the maintenance at the CO partial pressure of 0.6 atm, which is higher than the saturation concentration of CO for the bacterial growth (Fig. 3).

At the end of the culture the acetate concentration reached 90 mM. The rate of acetate production was higher than that of the bacterial cell growth at the later part of the fermentation. These results indicate that the

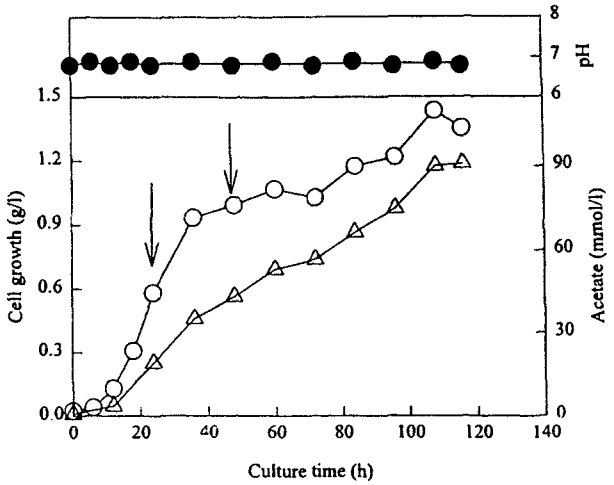

Fig. 6. The growth of *E. limosum* KIST612 with CO refreshment.

The gas-lift reactor was used to cultivate the bacterium, and the gas-tight bag was changed with fresh CO as indicated by arrows with the measurements of optical density (○), acetate concentration (△). pH (●) was controlled to 6.8 by addition of 2 N NaOH.

bacterium invests more maintenance energy requirement with acetate accumulation.

Effects of Refreshment of Medium Constituents during the Culture

The gas-lift reactor was used in another experiment with a 11.82 liter gas-tight bag to sustain the bacterial growth for a longer period of time. The bacterial cell concentration reached 1.7 g/l before decreased gradually (Fig. 7). After the cell concentration started to decrease, the pH controller was inactivated and solutions used for PBBM

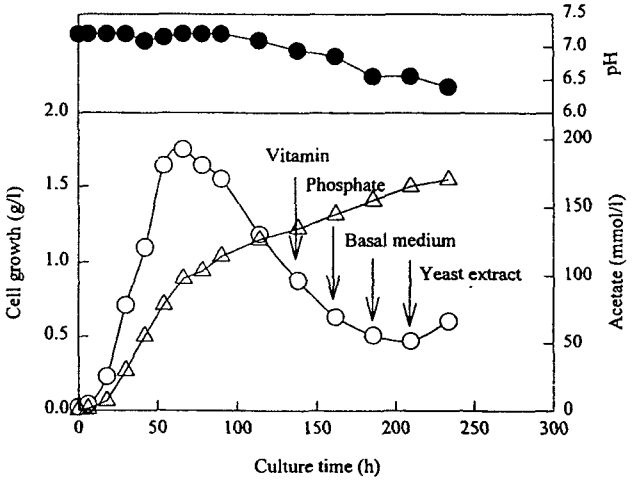

Fig. 7. The effects of PBBM constituents refreshments on the growth of *E. limosum* KIST612.

The gas-lift reactor with 11.82 litre gas-tight bag was used. When the cell concentration decreased, the culture was added by the solutions used to make up PBBM in sequence. pH (●) was not controlled with the additions.

were added sequentially to see if the medium constituents were limiting. As shown in Fig. 7, only yeast extract was effective in getting the bacterium to grow again. During the death phase of the culture, acetate was produced at a reduced rate. These results show that inorganic constituents of the PBBM are limiting the bacterial growth under these conditions, and that the energy available from the acetate formation at the reduced rate in the presence of acetate is not enough to support the chemolithotrophic bacterial growth.

DISCUSSION

The specific growth rate of *E. limosum* KIST612 was not consistent when CO was used as the sole carbon and energy source. It is not unusual to observe inconsistent growth rates in anaerobic bacteria. *E. limosum* KIST612 has been isolated and selected for its high growth rate under high CO concentrations. The maximum growth rate of 0.25 h^{-1} is not as high as *Peptostreptococcus productus*, but *P. productus* is initially inhibited by CO of partial pressure over 80 kPa (0.85 atm) [21]. Since CO limitation was observed with high cell concentration when the CO partial pressure was less than 0.6 atm, *P. productus* is not suitable for use in a process to convert CO-rich gases into multi-carbon compounds. The growth rate of *E. limosum* KIST612 was higher than those of other homoacetogens except *P. productus*.

Unlike typical homoacetogens, *E. limosum* is known to produce butyrate, ethanol and butanol besides acetate under certain conditions. These compounds are produced 1) to detoxify acetate in low pH, and 2) to dispose of excess reducing equivalent [22]. Butyrate yield was higher in the culture without pH control. It might be of interest to know if the excess electron also stimulates butyrate production by the bacterium. Interestingly, the bacterium produced a trace of isobutyrate. It is not clear at this stage if isobutyrate is produced from the oxidation of amino acids supplied as yeast extract or from the isomerization of butyrate, which is known in anaerobic ecosystems [24]. This result suggests that CO can be converted to alcohols or acids by careful manipulation of culture conditions.

It was shown that CO was limiting during the growth of *E. limosum* KIST612 when the CO partial pressure was less than 0.6 atm (60.8 kPa) even in reduced growth due to the accumulated acidic products. The K_s value for CO in the bacterium was measured to be 0.14 mM. The limitation was not due to the low affinity of the bacterium for CO, but due to the slow CO transfer rate into the liquid phase. Syn-gas and off-gas from a steel mill contain up to 65% (v/v) CO [9]. In order to use these gases for producing multi-carbon compounds, a pressurized reactor is required to avoid substrate limitation.

It is well known that acetate inhibits bacterial growth by increasing the maintenance energy. An *in situ* acetate removal process is required for a high rate of CO fermentation. This can be achieved in various ways. One of the options is membrane technology, which requires a pressure drop across the membrane. A pressurized reactor can improve the gas transfer rate as well as separation efficiency of the membrane.

Maintenance of anaerobic conditions is a problem associated with handling anaerobic microorganisms in a laboratory. General anaerobic procedures are well documented [19], but this communication is the first to describe an anaerobic system which uses a collapsible gas-tight bag as the container of the gaseous substrate to avoid oxidation of an anaerobic system due to the negative pressure caused by the consumption of the substrate.

Acknowledgments

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