Anaerobic CO_2 Fixation by the Acetogenic Bacterium *Moorella thermoacetica*

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Anaerobic bacteria such as Moorella thermoacetica have the capacity of fixing carbon dioxide with carbon monoxide and hydrogen for the production of ethanol, acetic acid, and other useful chemicals. In this study, we evaluated the fixation of CO_2 for the production of acetic acid, as a product in its own right but also as precursor for lipid synthesis by oleaginous organisms. We achieved maximum cell optical density of 11.3, acetic acid titer of 31 g/L, and productivity of 0.55 g/L-h at CO mass-transfer rate of 83 mM/h. We also showed electron availability by CO mass transfer limited the process at CO mass transfer rates lower than 30 mM/h. Further enhancement of mass-transfer rate removed such limitations in favor of biological kinetics as main limitation. This work underlines the potential of microbial processes for converting syngas to fuel and chemical products in processes suitable for distributed feedstock utilization. © 2013 American Institute of Chemical Engineers *AIChE J*, 59: 3176-3183, 2013
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Introduction

A gas feedstock platform for the synthesis of fuels and chemicals

Production of biofuels and bio-based chemicals from renewable resources has been traditionally considered in the context of a sugar platform, whereby the cellulosic and hemicellulosic components of biomass are deconstructed via pretreatment and enzymatic hydrolysis to the constituent simple sugars which are subsequently fermented by a suitable microbe to the final product of interest. This process, depicted in Figure 1a, is the preferred route for the production of cellulosic ethanol as well as other products. As shown in Figure 1a, the sugars platform includes sugars from sugarcane and corn starch hydrolysis, while microbes catalyzing the conversion of sugars may be natural yeast or bacteria, or engineered ones properly modified for the utilization of the five carbon sugars resulting from hemicellulose hydrolysis and also the production of products other than ethanol, such as isobutanol, butanediol, succinic acid, and others.[1,2]

An alternative to the sugar platform is the gas platform depicted in Figure 1b. Here, synthesized gas or gas mixtures of CO_2, CO, and H_2 are converted microbially to a variety of products. This biological route is normally referred to as syngas fermentation. The gas feedstock may be effluents of cement or steel manufacturing or the product of coal, methane, or biomass gasification. Products formed are typically ethanol and acetic acid.[3] Numerous other products can be formed as well by properly engineering the anaerobic organisms that catalyze the gas conversion, as indicated. Although Fischer–Tropsch synthesis can also be used for converting CO_2 and a reducing gas-to–liquid (GTL) fuels, such processes suffer from low selectivities and require large plants and capital investments to reduce product costs by taking advantage of economies of scale. On the other hand, microbial processes have typically very high selectivity thus requiring much smaller plants and capital investments. As such, they are better suited for utilization of distributed gas or renewable feedstock and production of liquid fuels and other chemical products. Additionally, biological syngas fermentation processes do not require high temperature and pressures, operate closer to equilibrium and are, consequently, more efficient with higher overall product yields.[4,5]

Several anaerobic acetogenic bacteria, such as *Moorella thermoacetica*,[6] *Clostridium ljungdahlii*,[7] and *Clostridium ragsdalei*,[8,9] are capable of conducting syngas fermentation. Acetogenic bacteria fix CO_2 via the Wood–Ljungdahl (W–L) metabolic pathway as shown in Figure 2. The pathway comprises two branches, the methyl branch and the carbonyl branch, by which CO_2 and CO as carbon sources, are converted into acetic acid and ethanol. In the methyl branch, one CO_2 molecule is converted, via formate, to a methyl moiety aided by the cofactor tetrahydrofolate. Reduction of carbon dioxide to the methyl group requires six electrons. In the carbonyl branch, two electrons are utilized to reduce one CO_2 molecule to CO, which reacts with the methyl moiety to form acetyl-CoA in a reaction catalyzed by the acetyl-CoA synthase enzyme. CO can also be used directly to form acetyl-CoA, rather than being produced from CO_2 reduction. Acetic acid, ethanol, and cell mass are the main products produced from the intermediate acetyl-CoA.[10]

Syngas fermentation is electron intensive. Electrons required for the metabolic process are derived from either

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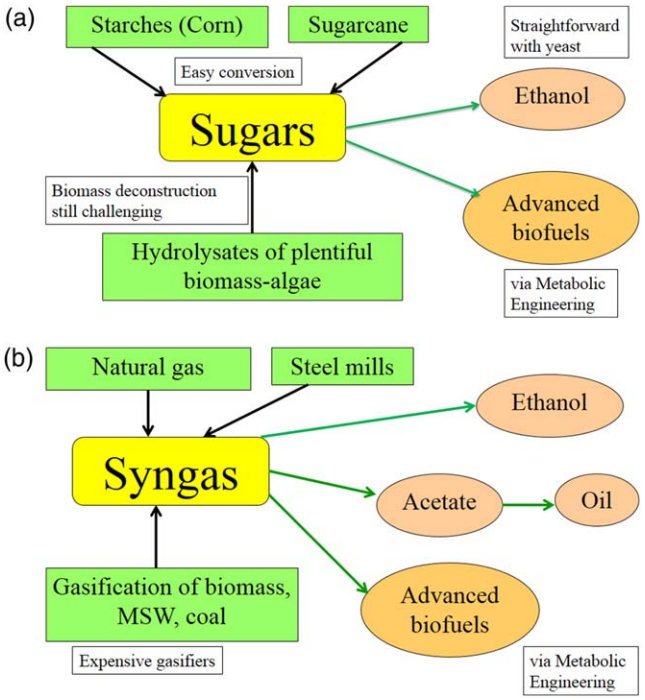

Figure 1. Two different platforms for biofuels production.

(a) Sugar platform. (b) Syngas platform. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

H_2 via the hydrogenase enzyme and/or from CO via the CO dehydrogenase (CODH) enzyme.[10] In all situations, eight moles of electrons, either from CO or H_2, are required for producing one mole of acetic acid. If all electrons are derived from H_2, the overall reaction associated with acetic acid production is

$$2CO_2 + 4H_2 \rightarrow C_2H_4O_2 + 2H_2O \quad (1)$$

If all electrons are derived from CO, the overall reaction becomes

$$4CO + 2H_2O \rightarrow C_2H_4O_2 + 2CO_2 \quad (2)$$

It is also important to note that, when acetate is the only product, the net ATP synthesis is zero. One ATP is consumed in methyl-branch and one ATP is produced in the conversion of acetyl-CoA to acetate (Figure 2). Consequently, ATP required for cell growth must be generated by pathways or mechanisms other than the W–L pathway. Two chemiosmotic mechanisms have been proposed, whereby either a proton gradient is generated to synthesize proton-dependent ATP production or a sodium gradient is generated to synthesize sodium-dependent ATP. The proton-gradient mechanism has been shown to involve an electron transport system and has often been associated with the presence of enzymes which generate electrons.[11] Thus, electron production from H_2 and CO can contribute to ATP production.

Production of fuel ethanol from syngas fermentation has recently received great industrial interest. A few companies, such as Coskata and Lanza Tech, were formed based on the commercialization of syngas fermentation. Currently, these companies are in the pilot-plant demonstration stage for ethanol production.[12] Besides generating fuel-level ethanol as the final product, syngas fermentation also produces acetic acid, which is valuable for plastics, film, food preservatives, and many other chemical industries.[13] More interestingly, as it can be seen in Figure 2, both ethanol and acetate are formed from acetyl-CoA, a key intermediate in cellular metabolism. Many other chemical products, such as butanol, lipids, biopolymers, and others can be biologically derived from acetyl-CoA.[14,15] As soon as a basic molecular biology tool kit for acetogenic organisms such as *M. thermoacetica* is available, the concepts and methods of metabolic engineering can be deployed toward the synthesis of any of the above and many other products. This could lead to a platform technology for biological GTL, or BioGTL, conversion. In light of the vast amounts of natural gas recently discovered, a Bio-GTL platform could be of great importance in advancing production of fuels and chemical products from abundant renewable (biomass) and natural gas feedstock.

In a variation of this concept, another approach for converting CO_2 and reducing gases to biofuels is a two-stage fermentation system in which acetic acid is produced from CO_2 in the first stage and is used as substrate for lipid production by an oleaginous organism in the second stage.[16] This scheme is capable of converting gases to natural oils, which can be upgraded to hydrocarbon fuels suited for use as liquid fuels in the current infrastructure.

In this work, we choose *M. thermoacetica* as the model microorganism. It is a gram-positive thermophilic anaerobe with relatively high acetate productivity and high gas utilization efficiencies. Sakai et al.[6] reported maximum of 20 g/L of acetic acid (339 mM) production from a mixture of CO_2 and H_2 after 220 h of fermentation using *Moorella* sp. HUC22-1. Also, unlike other acetogens such as *C. ljungdahlii* and *C. ragsdalei*, which also produce ethanol, *M. thermoacetica* produces acetic acid as the only end product. This makes it a perfect candidate to make acetate as feedstock for

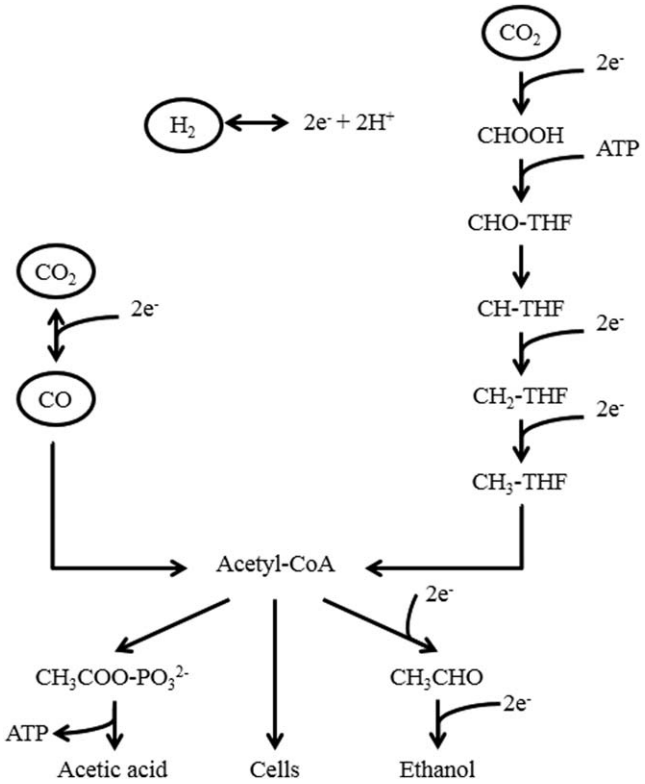

Figure 2. Wood–Ljungdahl (W–L) metabolic pathway for syngas fermentation (Adapted from Ljungdahl 1986[10]).

liquid biofuel production in a two-stage fermentation process as described above. Moreover, the genome of *M. thermoacetica* has been completely sequenced,[17] which can greatly facilitate the development of molecular biology tools for genetic engineering of the bacterium.

The GTL mass-transfer rate is known to impact the productivity of syngas fermentation.[18] Several studies have investigated various methods for enhancing the GTL mass-transfer rate. Generally, GTL mass transfer rate can be enhanced by increasing gas solubility via pressurizing the bioreactor or by increasing the GTL mass-transfer coefficient (k_La) via improving the bioreactor design. The mass-transfer coefficients depend on the operational parameters such as bioreactor geometry, gas flow rate, gas bubble size, agitation speed, and so forth. Most reported studies regarding syngas fermentation applied the continuous stirred-tank reactor (CSTR) because it is readily available. However, the bubble column bioreactor generally has much higher mass-transfer coefficient than that in the CSTR at same gas flow rate and same gas bubble size.[18–22] Other advantages of column bioreactor include simple construction, and no agitation required. The objective of this work was to study the feasibility of carbon dioxide fixation by CO and/or H_2 by *M. thermoacetica* in a bubble column bioreactor, and to assess mass-transfer limitations in the overall conversion process. We found that significant amounts of acetic acid can be produced by *M. thermoacetica* fermentation of syngas. Our results show that initial gas mass-transfer limitations can be overcome by increasing the volumetric mass-transfer coefficient of carbon monoxide leading to marked increases in the volumetric productivity of acetic acid production. Along with identifying carbon monoxide dehydrogenase as target for genetic modulation, these results underline the promising opportunities that exist in using gas platform microbial technologies for the production of fuels and chemicals through the fixation of carbon dioxide with CO and/or hydrogen as reducing substrates.

Materials and Methods

Bacterium and cultivation medium

The anaerobic acetogenic bacterium *Moorella thermoacetica* (ATCC 49707) (http://www.atcc.org) was used in this study. An enhanced culture medium containing minerals, trace metals, yeast extract, and reducing agent cysteine was used for the preparation of the inoculum and cell cultivation. Cells were grown under anoxic conditions following methods that preserve strict anoxic environment in the bioreactor. The medium (per liter) contained 1.4 g KH_2PO_4, 1.1 g K_2HPO_4, 2.0 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4{\cdot}7H_2O$, 10 g yeast extract, 10 g morpholinoethanesulfonic acid, 20-mL ATCC 1754 PETC trace elements solution (http://www.atcc.org), and 10 mL of 3% cysteine solution. In addition, 0.5 mL of 0.2% resazurin, a redox potential indicator, was added to indicate the presence or absence of oxygen. The seed culture used for inoculation of bioreactors was prepared in serum bottles using the media described above, following strict anoxic techniques. Yeast extract was purchased from BD (Franklin Lakes, NJ). All other chemicals used were purchased from Sigma-Aldrich (St. Louis, MO).

Bubble column bioreactor

A glass bubble column bioreactor was designed for the study. The glass column had an inside diameter of 4.5 cm and a height of 80 cm (G. Finkenbeiner Inc., Waltham, MA). The total working volume was about 1 L. A bubble diffuser (pore size ~20 μm; Alita Industries, Arcadia, CA) was used for delivering small gas bubbles during the experiment. To adjust the gas compositions, a four-channel gas flow control system (H_2, CO, CO_2, and N_2) was built. The bioreactor temperature was maintained at 60°C using a heating blanket. The pH of the media was controlled using an Etatron DLX pH/ORP pump control system which was connected with a submersible pH electrode. The base used for pH control was 5-N sodium hydroxide, and the acid used was 5-N hydrochloric acid (Sigma-Aldrich, St. Louis, MO). The units for gas flow control, temperature control, and pH control were all purchased from Cole-Parmer (Cole Parmer, Vernon Hills, IL) and assembled in our laboratory.

Fermentation in bubble column bioreactor

A series of experiments at varying pH, gas compositions, and gas flow rates were conducted in batch liquid media and continuous gas flow in bubble column bioreactors. The bioreactors were sprayed thoroughly with 70% ethanol and put in a laminar flow hood under UV light (The Baker Company, Sanford, ME) overnight prior to the experiments. Prepared medium was autoclaved at 121°C for 25 min, transferred to the bioreactors, and was purged with oxygen-free N_2 gas overnight to remove all traces of dissolved oxygen (DO). Afterwards, the medium pH was adjusted to 6.0 unless otherwise stated, and syngas flow was initiated and continuously bubbled through the bioreactors. Temperature was maintained at 60°C, and 10 mL of anaerobically prepared cysteine solution (3% w/w) was added to the medium to remove any residual oxygen. A 5% vol/vol inoculum of *M. thermoacetica* was inoculated to the bioreactor for the syngas fermentation experiments.

An initial experiment was run without pH control with initial pH of 7, and cells were grown on 70% CO and 30% CO_2 gas mixture at a total gas flow rate of 100 sccm (standard cubic centimeters per minute). Two other sets of pH-controlled experiments were conducted to evaluate the pH effects at controlled pH 7 and pH 6 at the same gas flow rate of 100 sccm and gas composition of 40% CO, 30% CO_2, and 30% H_2. Moreover, gas flow rates were varied such as to evaluate the effect of gas flow rates at 100, 200, and 1000 sccm, and at controlled pH 6 and gas composition of 70% CO and 30% CO_2.

Gas analysis

The flow rates and compositions of off gas from bioreactors were determined to assess the gas utilization at various situations. The flow rate was measured by bubble flow meter (Cole-Parmer, Vernon Hills, IL). Analysis of gas composition was conducted using a dual-channel Agilent micro-GC equipped with PLOT U and molecular sieve columns, and TCD detectors (Agilent Technology, Santa Clara, CA). Argon was used as the carrier gas.

Liquid analysis

At various time intervals, 1 mL of culture was sampled from the bioreactors and analyzed for cell optical density (OD) and acetic acid concentration. Samples were collected in 1-mL cuvettes, and the OD was measured at 600 nm using an Ultrospec 2100 pro UV/visible spectrophotometer (General Electric, Fairfield, CT). The OD was proportional

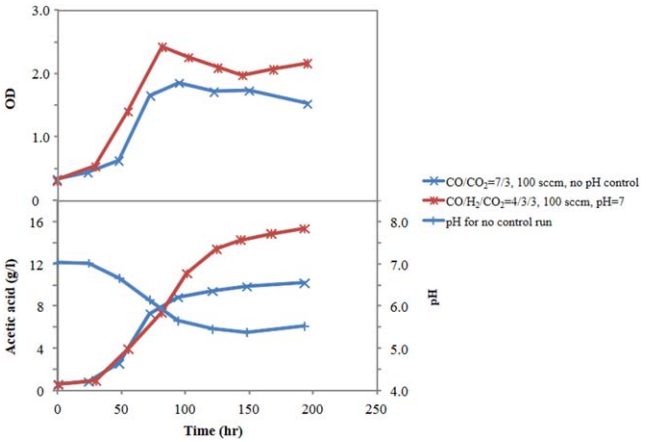

Figure 3. Cell growth, acetic acid production, and pH profile of the experiment under controlled pH = 7 and the experiment without pH control.

Typical results from at least duplicate runs for each group are presented. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

to the dry cell concentration (~0.46 g-dry cell/L per unit OD).[6] After measuring OD, samples were centrifuged at 14,000 rpm for 10 min, and the cell-free supernatant was analyzed for acetic acid using a Waters Alliance 2695 HPLC with a Waters 410 differential refractometer and a Bio-Rad HPX-87H column (Waters Corporation, Milford, MA). The column was eluted at 35°C with 14 mM of sulfuric acid as the mobile phase with the flow rate of 0.6 mL/min.

Determination of mass-transfer coefficient (k_La)

The volumetric mass-transfer coefficient (k_La) of oxygen at 60°C was determined by measuring the time course of DO concentration during transient conditions.[23] The 1-L bioreactor containing cell-free media was purged with nitrogen until saturated, at which conditions DO was zero. The gas feed was then switched to oxygen, and the time course of the DO in the liquid was measured using a DO probe (Cole Parmer, Vernon Hills, IL). A mass balance yields the following equation

$$\frac{dC_t}{dt} = k_La(C^* - C_t) \tag{3}$$

From which one obtains after integration

$$\ln(C^* - C_t) = -(k_La)t + A \tag{4}$$

where, C_t is the DO concentration in the liquid at any given time t and C^* is the saturated DO concentration, and A is the integration constant. Equation 4 suggests that k_La for oxygen can be determined from a semilog time plot of the DO concentration.

After determining the mass-transfer coefficients of oxygen, k_La values for the experimental gas can be determined by the following relationship between k_La and the aqueous diffusivities (D) for different gas species i and j[24]

$$\frac{(k_La)_i}{(k_La)j} = \left(\frac{D_i}{D_j}\right)^{0.5} \tag{5}$$

Using diffusivities of 3.05×10^{-5} cm^2/s, 3.26×10^{-5} cm^2/s, 2.74×10^{-5} cm^2/s, and 6.48×10^{-5} cm^2/s, respectively, for O_2, CO, CO_2, and H_2 at 37°C, the k_La values for CO, CO_2, and H_2 were determined. It should also be noted that the ratio of diffusivities for different gas species keeps approximately constant with temperature due to Stokes–Einstein equation, though the diffusivity of the specific gas has temperature dependence.[25]

Results and Discussion

Moorella grows well on syngas and produces substantial amounts of acetate

M. thermoacetica growth and production of acetate as the only detectable end-product were observed in this study. Figure 3 shows the results for cell growth and acetic acid production for a non-pH-controlled fermentation and one for which pH was controlled at pH = 7. The maximum OD achieved, acetic acid titers, volumetric productivities (R), and specific productivities (specific R) of acetic acid during the main productive phase are tabulated and shown in Table 1. It can be seen that substantial amounts of acetic acid (in excess of 10 g/L) were produced from a rather dilute cell culture (OD of ~2). The rather high specific productivity is probably due to the large amounts of acetic acid required to generate rather small amounts of energy for growth by the chemiosmotic theory. Substantially, higher acetic acid level (~16 g/L) was achieved under pH control at pH = 7. Inspection of the pH profile of the run without pH control suggests that acidity below pH value of 5.5, perhaps in combination with the high acetic acid concentrations, inhibits cell growth and product formation. Although these inhibitory effects were reduced under pH control at pH = 7, this came at a cost of a prolonged product formation phase which increased the acetic acid titer, but did not result in higher overall productivity of the pH-controlled fermentation compared to the one without pH control (Figure 3).

Similar experiments comparing productivity and acetate level under controlled pH identified pH = 6 as a better set

Table 1. The Maximum Cell Density, Acetic Acid Titer, Volumetric Productivity, and Specific Productivity of Acetic Acid During the Main Productive Phase at Varying Experimental Conditions

Run	Maximum OD	Acetic Acid Titer (g/L)	R (g/L-h)	Specific R (h^{-1})
CO/CO_2 = 7/3, 100 sccm, no pH control	1.8	10	0.20	0.39
$CO/H_2/CO_2$ = 4/3/3, 100 sccm, pH 7	2.4	16	0.12	0.11
$CO/H_2/CO_2$ = 4/3/3, 100 sccm, pH 6	2.5	26	0.13	0.16
CO/CO_2 = 7/3, 100 sccm, pH 6	1.8	29	0.21	0.40
CO/CO_2 = 7/3, 200 sccm, pH 6	4.3	31	0.30	0.16
CO/CO_2 = 7/3, 1000 sccm, pH 6	11.3	30	0.55	0.14

The volumetric productivity and specific productivity were calculated during the main productive phase: (a) from 48 to 72 h; (b) from 55 to 143 h; (c) from 48 to 192 h; (d) from 48 to 144 h; (e) from 42 to 112 h; (f) from 51 to 89 h.

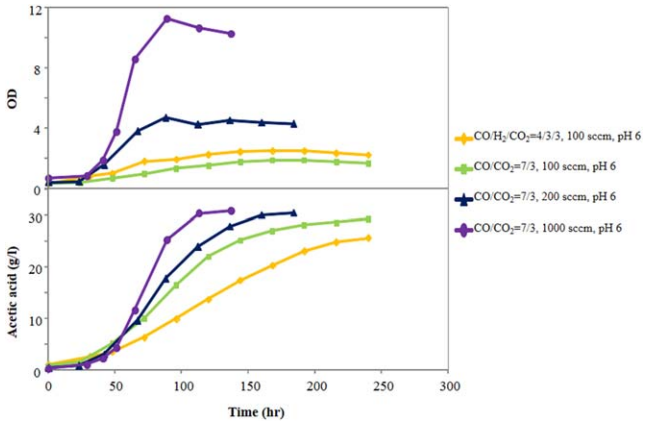

Figure 4. Cell growth and acetic acid production at various gas compositions and flow rates under controlled pH=6.

Typical results from at least duplicate runs for each group are presented. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

point for pH control that maximized acetic acid productivity (Figure 4).

Acetic acid productivity increases with gas flow rate

The next series of experiments investigated the effect of gas flow rates and compositions under controlled pH = 6 (Figure 4). First, the run at flow rate of 100 sccm and gas composition of 40% CO, 30% CO_2, and 30% H_2, is directly comparable to the run conducted under identical conditions but pH = 7, shown in Figure 3. A maximum OD of 2.5 and volumetric productivity of 0.13 g/L-h were obtained, very similar with the results obtained under pH = 7. However, although at the higher pH the run ended after 150 h with a product concentration of about 16 g/L, at pH = 6, a product titer of 26 g/L was obtained after about 250 h. This indicates that the effect of more acidic pH is to increase cell tolerance to acetate but not to affect the productivity of the cells to produce acetic acid.

Next, we compared the efficiency of CO and H_2 as electron sources for the fixation of CO_2. To this end, a fermentation was conducted at controlled pH 6 with a gas flow rate of 100 sccm but the gas composition was changed to 70% CO and 30% CO_2. Compared to the previous run carried out at the same gas flow rate and pH, this experiment simply substituted H_2 as electron source with carbon monoxide. It can be seen that, with CO as electron source, the acetic acid volumetric productivity (0.21 g/L-h) was substantially higher than the case where H_2 was used (0.13 g/L-h) (Table 1). Because the total supply of available electrons was kept the same in the two runs, the observed productivity difference suggests that CO is a more efficient electron source than H_2, as far as acetic acid synthesis is concerned. It is noted that the differences in productivity were not accompanied by commensurate changes in cell concentration, which were rather similar in the two runs. It appears that acetic acid productivity is not necessarily related to cell density or cell growth rate.

Finally, we studied how gas flow rate affects productivity by increasing the gas flow rates to 200 and 1000 sccm, respectively, while keeping the other experimental conditions the same (controlled pH 6 and gas composition of 70% CO and 30% CO_2). The results showed that higher flow rate resulted in increased cell growth rate and faster acetate production (Figure 4). A maximum OD of 11.3 was observed within 89 h at gas flow rate of 1000 sccm, which was several times higher than that obtained in the other runs. Similarly, 30 g/L of acetic acid was produced after 113 h, resulting in a maximum volumetric productivity of 0.55 g/L-h in this run. The productivity was higher than Sakai's study, in which 20 g/L of acetic acid was produced after 220 h.[6]

Clearly, volumetric productivities of acetic acid are directly related to the gas composition and gas flow rate. However, the sharp increase in volumetric productivity resulted from an increase in total cell density rather than specific acetic acid productivity. The latter actually decreased significantly at the high gas flow rate, owing to the sharp increase in total cell density (Figure 4 and Table 1). It appears that cell growth, volumetric productivity, and specific productivity are not strictly related. One possible reason could be the energy generation system of these microorganisms where ATP production depends on the proton-gradient mechanism and not on the metabolic pathway of acetic acid synthesis itself. This would make cell growth independent of acetic acid production. At the same time, proton gradients depend on the electron-transfer processes from hydrogen and CO, and the efficiency by which ATP is generated by electrons transferred through the two key enzymes, hydrogenase and CODH, respectively.[11]

The rate of acetate production is mass transfer limited

The data of Figure 4 can be placed in perspective by considering gas mass-transfer effects. The GTL mass-transfer rate is expressed as

$$r_i = (k_L a) \times \left(f \times C_i^* - C_{i,t}\right) \times V \tag{6}$$

where subscript i represents the gas species in the syngas mixture, and r_i is the mass-transfer rate of species i (in mole/h), f_i is the fraction of species i in the gas mixture, C_i^* is the solubility of species i under one atmosphere pressure at the given temperature, $C_{i,t}$ is the dissolved concentration of species i at any given time point t, and V is the working volume of the bioreactor, which is 1 L in this study.

A maximum mass-transfer rate ($r_{i,max}$) is achieved when the process is mass transfer limited in which case the concentration of species i ($C_{i,t}$) is zero (Eq. 7)

$$r_{i,\max} = (k_L a) \times \left(f_i \times C_i^*\right) \times V \tag{7}$$

Equation 7 indicates that the maximum mass-transfer rate of species i (CO_2, CO, and H_2) is directly proportional mass-transfer coefficient, fraction, and the gas solubility. Mass-transfer coefficient can be obtained from the measured $k_L a$ of O_2 using the diffusivity correlation. $k_L a$ values for oxygen were experimentally determined at gas flow rates of 100, 200, and 1000 sccm, respectively, at 60°C in the designed bubble column bioreactor and are shown in Table 2. Solubility values for CO_2, CO, and H_2 were obtained from the literature as 1.4×10^{-2}, 5.6×10^{-4}, and 6.2×10^{-4} M at one atmosphere at 60°C.[26] It should be noted that, because the values of the mass-transfer coefficients of CO_2, CO, and H_2 do not differ very much, high gas solubility of CO_2, which is one order of magnitude higher than CO and H_2, would yield a much faster mass-transfer rate for CO_2 relatively to those of CO and H_2. This makes it very unlikely that the mass-transfer rate of the carbon source, CO_2, would be the limiting step of the process. We thus focused on possible rate limitation by electron availability due to carbon monoxide mass-transfer limitation.

Table 2. A Summary of Mass-Transfer Coefficients of O_2, CO_2, CO, and H_2 in 1-L Bubble Column Bioreactor at 60°C

Flow (sccm)	k_L a O_2 (h^{-1})	k_L a CO_2 (h^{-1})	k_L a CO (h^{-1})	k_L a H_2 (h^{-1})
100	48.0 ± 2.3	45.5	49.5	70.0
200	73.2 ± 0.6	69.4	75.7	106.7
1000	204.0 ± 2.1	193.4	210.9	297.3

The values of mass-transfer coefficients of O_2 represent the averages and standard deviations from triplicate experiment.

The maximum cumulative number of available electrons (in moles) from carbon monoxide at time t ($E_{CO,max}$) can be determined from the following equation (note that two moles of electron become available for each mole of CO transferred)

$$E_{CO,max} = (k_L a)_{CO} \times \left(f_{CO} \times C^*_{CO}\right) \times V \times \text{time} \times 2 \qquad (8)$$

The above maximum electron availability can be compared to the moles of electron recovered in the newly synthesized biomass and acetate moles measured in the medium. Using a molecular formula for cell biomass of $CH_{2.08}O_{0.53}N_{0.24}$, (molecular weight = 26 g/mole),[27] and a degree of reductance of 4.3, the total electron moles captured in cell biomass of OD is: 4.3 × OD × 0.46/26. In addition, to produce one mole of acetic acid ($C_2H_4O_2$, molecular weight 60 g/mole) from syngas, eight moles of electrons are required. Thus, the total amount of electron moles captured in cell biomass and acetate can be expressed as

$$\text{Exp}_E = (4.3 \times \text{OD} \times 0.46/26 + 8 \times C_{\text{aceticacid}}/60) \times V \qquad (9)$$

Equations 8 and 9 allow a direct comparison between the cumulative electrons captured in cell biomass and acetate in the course of the experiment and the maximum available from CO. Figure 5 shows this comparison for the experiments conducted at pH 6 starting 30 h after the initiation of the experiment to exclude the lag phase. The results include the runs conducted at 100 and 200 sccm and varying gas composition. It can be seen that, at gas flow rates of 100 sccm and 200 sccm, the total amount of captured electrons are in close agreement with the calculated maximum available electrons under mass-transfer limitation, indicating that

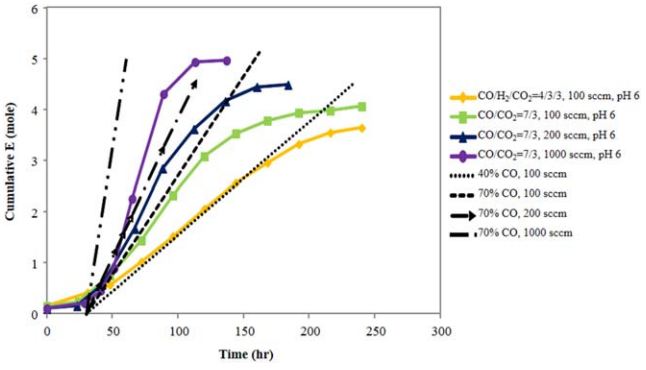

Figure 5. The comparison between the utilized electrons calculated from experimental results and the maximum available electrons from carbon monoxide calculated from mass transfer modeling.

[Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

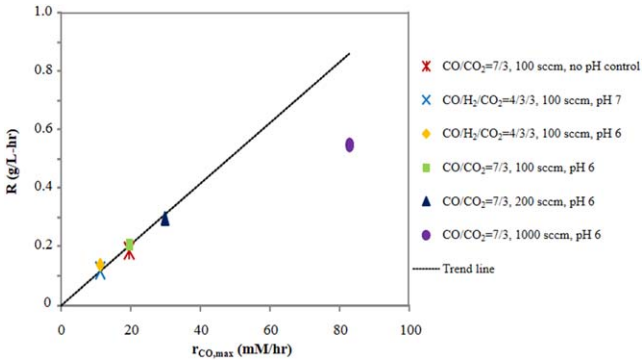

Figure 6. The correlation between the volumetric productivity (R) of acetic acid and the maximum mass transfer rate of carbon monoxide ($r_{CO,max}$).

The straight line represents the trend of the first five points in which experiments were conducted with flow rate of 100 and 200 sccm. The fitted equation is $Y = 0.0104\times$. $r^2 = 0.982$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the fermentation was indeed limited by the mass-transfer rate of CO.

Figure 6 shows the direct correlation between the maximum mass-transfer rate of carbon monoxide $r_{CO,max}$ (calculated from Eq. 7) and the acetic acid productivity. At values of $r_{CO,max}$ below 30 mM/h, acetic acid productivity is proportional to the maximum mass-transfer rate of CO. The good linear fit again confirms that syngas fermentation under these experimental conditions was solely limited by the availability of carbon monoxide.

Acetic acid production becomes biologically limited at very high gas mass-transfer rates

To test the limit of the correlation of Figure 6, another run was carried out at a very high gas flow rate of 1000 sccm. It can be seen that, under these conditions, the data points representing total electrons captured in the process are below the maximum calculated from mass-transfer limitations (Figure 5). This is further exemplified by the correlation of Figure 6 showing that at higher CO mass-transfer rate ($r_{CO,max}$ = 83 mM/h, corresponding to the experiment with 1000 sccm and gas composition of 70% CO and 30% CO_2), acetic acid productivity is no longer proportional to the maximum CO mass-transfer rate. Thus, at conditions of such high gas flow rate and CO mass transfer rate, the fermentation is no longer limited by the GTL mass transfer.

The mass-transfer limitation at low gas flow rates explains why higher cell mass densities did not result in higher volumetric productivity of acetic acid. Despite variations in cell density, the rate at which cells can produce acetic acid is limited by the rate of CO_2 fixation which is in turn limited by electron availability and CO mass transfer. Consequently, higher cells mass density did not necessarily result in higher volumetric productivity.

Electrons are supplied in these experiments by two possible sources, hydrogen and carbon monoxide. At the same gas flow rate of 100 sccm and same gas composition of 40% CO, 30% CO_2, and 30% H_2, but different pH (controlled pH 6 or 7), similar acetic acid productivities of 0.12 g/L-h and 0.13 g/L-h were obtained. However, when hydrogen was

Table 3. Gas Substrate Utilization and Product Yields in the 1-L Bubble Column Bioreactors at Various Gas Compositions and pH Levels While Maintaining the Same Inlet Gas Flow Rate of 100 sccm

Run	Culture Time (h)	Dry Cells (g)	Acetate (mole)	CO Consumed (mole)	H_2 Consumed (mole)	Cell Yield (g/mole-CO)	Acetate Yield (mole/mole-CO)
$CO/H_2/CO_2 = 4/3/3$, pH = 7	120	0.92 ± 0.20	0.21 ± 0.02	0.98 ± 0.04	0.05 ± 0.03	0.95 ± 0.23	0.22 ± 0.01
$CO/H_2/CO_2 = 4/3/3$, pH = 6	120	0.83 ± 0.12	0.22 ± 0.02	1.10 ± 0.10	0.05 ± 0.02	0.77 ± 0.15	0.20 ± 0.01
$CO/CO_2 = 7/3$, pH = 6	120	0.87 ± 0.07	0.35 ± 0.03	1.54 ± 0.13	NA	0.55 ± 0.07	0.23 ± 0.01

Values represent the averages and standard deviations from triplicate experiment.

replaced by CO (pH 6, 100 sccm, 70% CO, and 30% CO_2), a significantly higher productivity of 0.21 g/L-h was obtained. These results suggest that electron availability via hydrogenase is inhibited and that the productivity of acetic acid is solely affected by the availability of CO.

We analyzed the gas flow rate and composition for the bioreactor runs at various gas compositions and pH levels while the total gas flow rates were maintained at 100 sccm (Table 3). It was observed that in runs with all three gases (100 sccm, 40% CO, 30% CO_2, and 30% H_2, controlled pH 6 or 7), CO was consumed, whereas H_2 amount remained almost constant in the inlet and outlet gas stream. This observation indicates that in the presence of both CO and H_2, the electrons are derived only from CO, which is in accordance with previous reports on hydrogenase inhibition by CO. Several independent studies have shown that H_2 is not utilized when both CO and H_2 are present in the system.[28–30] As H_2 was not utilized in these experimental conditions, it was decided to not include calculation of biologically available electrons from H_2 in the discussion. Despite not being utilized in presence of CO, H_2, via hydrogenase, is an effective electron source for *M. thermoacetica* if the system does not contain CO, for example, a bioreactor running with a gas mixture of CO_2/H_2.[6] Care must be taken regarding the maximization of the feedstock utilization for designing a commercial syngas fermentation process. Table 3 also summarized the cell yield and acetate yield. For all three runs presented, the acetate yields over CO ranged between 0.20 and 0.23, which are close to the theoretical value of 0.25 calculated from Eq. 2. The high product yield is resulted from the high product specificity, and the relatively low cell mass required during syngas fermentation. The high efficiency of gas (CO) conversion during syngas fermentation is a potential advantage for commercial application.

The results obtained at 1000 sccm suggest that at sufficiently high mass transfer rates the mass-transfer limitation has been removed, and the biological kinetics becomes the limiting factor in carbon dioxide fixation and acetic acid production. The potential biological limitations like enzyme activities are required to be identified as the promising targets and the bottleneck enzymes should be overexpressed to further enhance the productivity of the process.

Conclusions

This study evaluated the mass transfer and kinetics of carbon fixation by the acetogenic bacterium *M. thermoacetica*. A maximum cell OD of 11.3, maximum acetic acid titer of 31 g/L, and highest acetic acid productivity of 0.55 g/L-h were achieved in a bubble column bioreactor. Further analysis showed that the rate of carbon fixation was proportional to the mass-transfer rate of carbon monoxide at CO mass transfer rates lower than 30 mM/h. At greater mass transfer rates, the process became biologically limited most likely by some key enzymes involved in the metabolic pathway. The latter could be a target of future metabolic engineering modulations aiming at further enhancing process productivity. Additionally, improving the microbe's acid tolerance should be another priority though the lower acetic acid levels may be also be accommodated by the production of intracellular products (like lipids by an oleaginous microorganism) or compounds that easily phase separate, in potential biofuel production applications.

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