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Utilization of CO_2 Fixating Bacterium *Actinobacillus succinogenes* 130Z for Simultaneous Biogas Upgrading and Biosuccinic Acid Production

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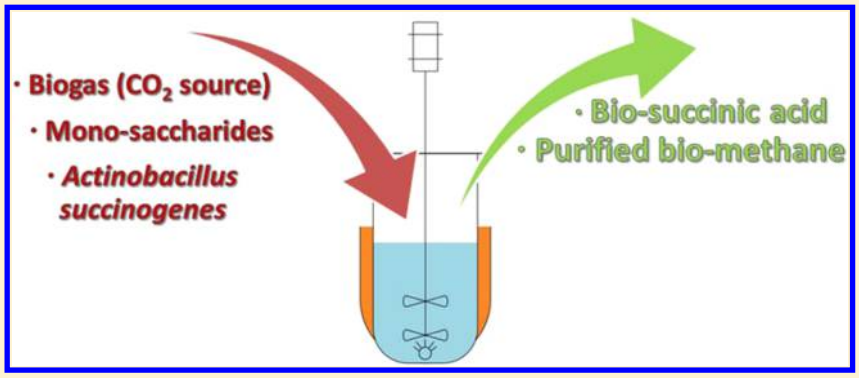
Supporting Information



ABSTRACT: Biogas is an attractive renewable energy carrier. However, it contains CO_2 which limits its use for certain applications. Here we report a novel approach for removing CO_2 from biogas and capturing it as a biochemical through a biological process. This approach entails converting CO_2 into biosuccinic acid using the bacterial strain *Actinobacillus succinogenes* 130Z, and simultaneously producing high-purity CH_4 (> 95%). Results showed that when pressure during fermentation was increased from 101.325 to 140 kPa, higher CO_2 solubility was achieved, thereby positively affecting final succinic acid yield and titer, CO_2 consumption rate, and CH_4 purity. When using biogas as the only CO_2 source at 140 kPa, the CO_2 consumption rate corresponded to 2.59 L CO_2 L^{-1} d^{-1} with a final succinic acid titer of 14.4 g L^{-1}. Under this pressure condition, the highest succinic acid yield and biogas quality reached corresponded to 0.635 g g^{-1} and 95.4% (v v^{-1}) CH_4 content, respectively, after 24 h fermentation. This work represents the first successful attempt to develop a system capable of upgrading biogas to vehicle fuel/gas grid quality and simultaneously produce biosuccinic acid, a valuable building block with large market potential in the near term.

INTRODUCTION

The EU Renewable Energy Source (RES) directive states that EU members should increase RES share of final energy consumption to an EU average of 20% and reduce CO_2 emissions 15−30% by 2020. With the EU commission trying to implement its emissions trading system (EU ETS),[1] firms are likely to be charged a carbon tax of €10 per metric tonne of CO_2 emissions by the end of 2014 (predicted to reach €13 by the end of 2015).[2] In the context of the EU directive framework, increased biofuel production and consumption should be complemented by increased production and use of biobased chemicals and should be preferred over their petrochemical derived equivalents.[3] Gaseous biofuels, mainly biogas, are increasingly becoming an important renewable energy source for combined heat and power (CHP) generation, while their role as transport fuel is also expected to increase in the coming years.[4] Biogas, produced through anaerobic digestion of organic material, is generally composed of 50−75% CH_4 and 25−50% CO_2. Depending on the final use, different processing steps are necessary. For instance, where it is important to have high energy content in the gas, as in vehicle fuel or for grid injection, the CH_4 needs to be purified (upgrading).[5] By removing CO_2 from the biogas in the upgrading process the energy content of the gas increases. Current upgrading technologies such as absorption make use of the physical/chemical properties of CO_2 where it is captured from the biogas (thereby increasing the CH_4 purity) and then released into the atmosphere, contributing to the global warming effect.[5] The necessity for reducing CO_2 emissions has never been greater, as recently the amount of CO_2 in earth's atmosphere was observed to exceed 400 ppm for the first time since measurements started 55 years ago,[6] also the fact that current technologies for CO_2 capturing and storage are costly, therefore processes that use CO_2 as a raw material are likely to become increasingly important.[7] An example of such a process is biological production of succinic acid through bacterial

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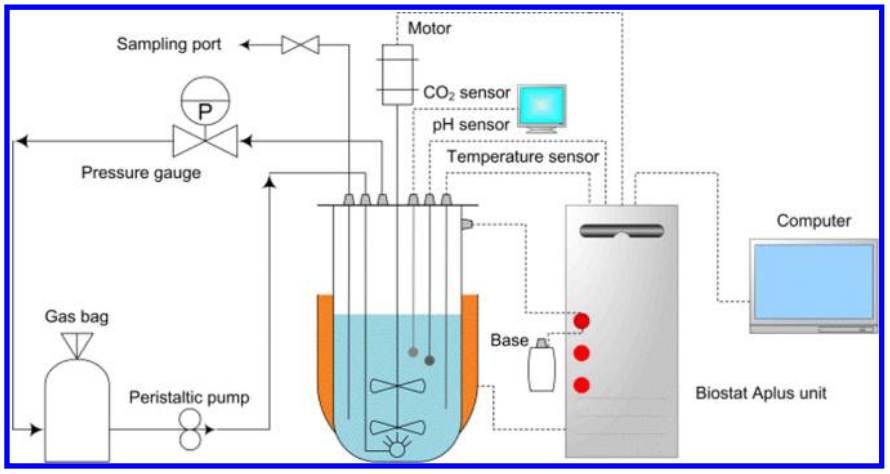

Figure 1. Experimental setup.

fermentation, where CO_2 fixation is performed by *Actinobacillus succinogenes* or any other bacterium capable of producing succinic acid.[8] Succinic acid is a four-carbon diacid with the chemical formula $C_4H_6O_4$ and is a precursor in the chemical synthesis of numerous commodities in agricultural, food, chemical, and pharmaceutical industries (e.g., solvents (1,4-butanediol, tetrahydrofuran, 2-pyrrolidinone, and gamma-butyrolactone) and biodegradable polymers (polybutylene succinate (PBS) and polyamides)).[8,9] Producing succinic acid biologically through fermentation has the advantage of consuming 1 mol of CO_2 per 1 mol of succinic acid produced, opposed to producing it chemically through hydrogenation of maleic acid as most of it is currently produced.[10] Substantial CO_2 emission savings in the range of 4.5−5 t per t biosuccinic acid produced have been estimated compared to emissions of petrochemical-derived succinic acid, therefore, it is evident that biosuccinic acid production could contribute to the abatement of CO_2 emissions.[11] A number of bacterial strains are able to produce succinic acid through fermentation, including *Actinobacillus succinogenes*,[12] *Mannheimia succiniciproducens*,[13] *Anaerobiospirillum succiniciproducens*,[14] *Corynebacterium glutamicum*,[15] and recombinant *Escherichia coli*.[16] *A. succinogenes* is recognized as one of the most promising microorganisms utilized for biosuccinic acid production, as it is a facultative anaerobe with high tolerance to initial sugar concentrations ($\leq$ 158 g L^{-1} D-glucose), but also tolerates high levels of succinic acid (> 70 g L^{-1}).[12,17] Additionally *A. succinogenes* has a broad preference of carbon sources such as glucose, xylose, arabinose, galactose, maltose, fructose, sucrose, cellobiose, lactose, mannitol, arabitol, sorbitol, and glycerol. This makes it possible to utilize a large variety of residual biomasses, such as carbohydrate-rich wastes or crude glycerol, a byproduct from biodiesel industry, for succinic acid production.[18−22] The fermentation end products of *A. succinogenes* are succinic acid as the main metabolite, in addition to formate, acetate, and ethanol.[12,23] The supply of CO_2 is a key factor in the production of succinic acid by *A. succinogenes*. A higher concentration of dissolved CO_2 in the fermentation broth could result in increasing the ratio of succinic acid to the other metabolites, the ratio of carbon recovery, and the succinic acid yield.[24] When using biogas as the only CO_2 source, the maximum dissolved CO_2 concentration in the culture broth corresponds to its solubility at the corresponding CO_2 partial pressure in the biogas (25−50% (v v^{-1})). However, one way to make CO_2 more available to the bacterium in the culture broth is to increase its solubility by means of increasing its partial pressure. This can be predicted by means of Henry's law. More complex models can also be used; the choice of model will strongly depend on what system operation conditions are imposed. A mathematical model was used together with experimental measurements to predict the dissolved CO_2 in culture broth. The model and parameters used in this work were retrieved from an earlier study unless otherwise stated (see Supporting Information (SI)).[25] In this study we demonstrate a new biogas upgrading technology, which makes use of bacterial fermentation to simultaneously produce high-purity CH_4 and biosuccinic acid. The benefits of integrating both processes—succinic acid production via bacterial fermentation and biogas upgrading—would potentially overcome the cost of CO_2 capture and storage and the cost of biogas upgrading, thereby promoting biobased economy.

EXPERIMENTAL SECTION

Chemicals and Gases. All chemicals used in this study were of analytical grade and purchased from Sigma-Aldrich ApS (Brøndby, Denmark); gases were supplied by AGA A/S (Copenhagen, Denmark).

System Setup and Operation. Two identical 3-L bioreactors (Sartorius BIOSTAT Aplus, Germany) with an initial liquid working volume of 1.5 L were used. The bioreactors were equipped with EASYFERM PLUS K8 200 pH probes (Hamilton Bonaduz AG, Switzerland), temperature probes (Sartorius, Germany) and InPro 5000i dissolved CO_2 sensors (Mettler Toledo, Switzerland). Flow rate of gas mixtures was controlled through a peristaltic pump (Watson-Marlow, United Kingdom). For monitoring and controlling pressure inside bioreactors a pressure gauge (Cewai, Italy) was mounted with a relief valve (Hy-Lok, USA) set to the experimental pressure. Gas−liquid ratio is defined in this study as initial volume of gas per liquid working volume. Experiments were conducted using different gas−liquid ratios (8.3:1 and 5:1). As shown in Figure 1 the gas was recirculated through the system during fermentation, allowing changes in biogas composition to be observed. Under anaerobic conditions, the biogas was injected through the sparger at the bottom of the reactor. As the biogas bubbles go through the reactor some of the gaseous CO_2 gets solubilized in the culture broth. As fermentation progresses, CO_2 decreases in the liquid phase (due to consumption by bacterial fermentation) which, according to Henry's law, forces more CO_2 to the gas phase to be dissolved into the culture broth. In this way the CH_4 content of the biogas is increased.

Microorganisms, Medium, and Succinic Fermentation. The strain of *Actinobacillus succinogenes* 130Z (DSM 22257) was obtained from DSMZ. The culture stock was stored in glycerol at −80 °C until used. Seed culture medium was composed of (g/L) glucose (10.0), yeast extract (5.0), $NaHCO_3$ (10.0), $NaH_2PO_4 \cdot 2H_2O$ (9.6), and $K_2HPO_4 \cdot 3H_2O$ (20.3). Medium was sterilized at 121 °C for 20 min. Seed culture was cultivated at 37 °C and 150 rpm in 50-mL sealed anaerobic bottles containing 30 mL of medium and inoculated with 1 mL of −80 glycerol stock culture. Biogas upgrading and succinic acid production batch experiments were conducted in duplicates. Fermentation experiments were conducted using biogas (60% CH_4, 40% CO_2) or 100% CO_2 as only source of CO_2 for succinic acid production. Fermentations were carried out at either 101.325 or 160 kPa pressure. Experimental medium for biogas upgrading and fermentation was composed of (g/L) yeast extract (10.0), K_2HPO_4 (3.0), $MgCl_2$ (0.2), $CaCl_2$ (0.2), and NaCl (1.0). Medium was sterilized at 121 °C for 20 min. Sterile glucose solution was added separately to the bioreactors to get a glucose concentration of 30−32 g L^{-1}. All batch fermentations in 3-L bioreactors were carried out at 37 °C, pH 6.75, and 200 rpm for 24 h, and inoculated with 5% (v v^{-1}) of exponentially growing inoculum. Prior to start of fermentation, pH was adjusted to 6.75 using 50% (w v^{-1}) phosphoric acid, and 0.05 mL of sterile Antifoam 204 (Sigma-Aldrich) was added. NaOH (8 M) was added automatically to maintain the pH at 6.75 during fermentation. N_2 gas was used in all cases to create anaerobic conditions prior to start of fermentation.

Analytical Methods. Dissolved CO_2 in fermentation broth was determined experimentally using an InPro 5000i (Mettler Toledo, Switzerland) CO_2 sensor controlled by M400 transmitter (Mettler Toledo, Switzerland). Gas containing 100, 40, and 20% (v/v) CO_2 was used to determine the dissolved CO_2 in fermentation broth at 101.325, 120, and 140 kPa pressure with temperature and pH fixed at 37 °C and 6.8, respectively. Cell growth was monitored by measuring the optical density at 660 nm (OD_{660}) using a spectrophotometer (Jenway Buch and Holm A/S 64050UV/vis). At OD_{660} of 1.0, *A. succinogenes* 130Z has a concentration of 0.626 g dry cell weight (DCW) L^{-1} (data no shown). Glucose, succinic acid, lactic acid, formic acid, acetic acid, and ethanol were all detected and quantified using high-performance liquid chromatography (HPLC). The HPLC (Agilent 1100 series) had a refractive index detector (detection of sugars, VFAs, and ethanol) and a Bio-Rad Aminex HPX-87H column (300 mm × 7.8 mm) with 4 mM H_2SO_4 as an eluent at a flow rate of 0.6 mL min^{-1} with column oven temperature set to 63.5 °C. To protect the HPX-87H column from contamination and foreign particles a guard column was fitted to the system. The yield of succinic acid, expressed as a percentage, was defined as the amount of final succinic acid produced from 1 g of reduced sugar consumed.

Model Description and Calculation. From low to moderate pressures at supercritical temperature, the solubility of a gas (e.g., CO_2) in a pure solvent (e.g., water) can be calculated by Henry's law:

$$p_{CO_2} = H_o C_{CO_2}$$

where p_{CO_2} is the CO_2 partial pressure in a gas mixture (kPa), H_0 is the Henry's constant for CO_2 in a pure solvent (kPa L mol^{-1}), and C_{CO_2} is the concentration of dissolved CO_2 in the liquid phase (mol L^{-1}). However, in a culture broth different kinds of organic substances and salts are always present which affects the solubility of CO_2. The effect of pH, temperature, and the organic substances and salts on CO_2 solubility has been taken into account through an empirical model for further predictions. For details see the Supporting Information.

Statistical Analysis. An ANOVA analysis followed by Fisher's Least Significant Difference test (LSD, $p < 0.05$) was used to evaluate if any significant differences were observed between model predictions and experimental measurements. All statistical analyses mentioned above were carried out using OriginPro software, version 9.0.0 (OriginLab Corporation, Northampton, MA).

RESULTS AND DISCUSSION

Proof of Concept. In this experiment, biogas (60% CH_4, 40% CO_2) was used as the only source of CO_2 for succinic acid fermentation (Figure 2a) using a gas−liquid ratio of 8.3:1. At atmospheric pressure (CO_2 partial pressure of 40 kPa), the maximum solubility of CO_2 corresponds to 9.15 mM. As shown in Figure 2b at 101.325 kPa, the final biogas composition at the end of the fermentation corresponded to 76.4% CH_4, 24.6% CO_2. The CO_2 partial pressure lessens as CO_2 is consumed during the course of the fermentation. This resulted in a decrease of the solubility of CO_2 (5.4 mM) in the culture broth at the end of the fermentation. In terms of succinic acid production, the final titer was 12.85 g L^{-1} with a yield of 0.60 g g^{-1}. When fermentation was performed at 101.325 kPa, the overall CO_2 consumption rate during 24 h fermentation corresponded to 1.35 L CO_2 L^{-1} d^{-1}. Succinic acid yield and productivity were in agreement with those reported in previous studies.[26] To our

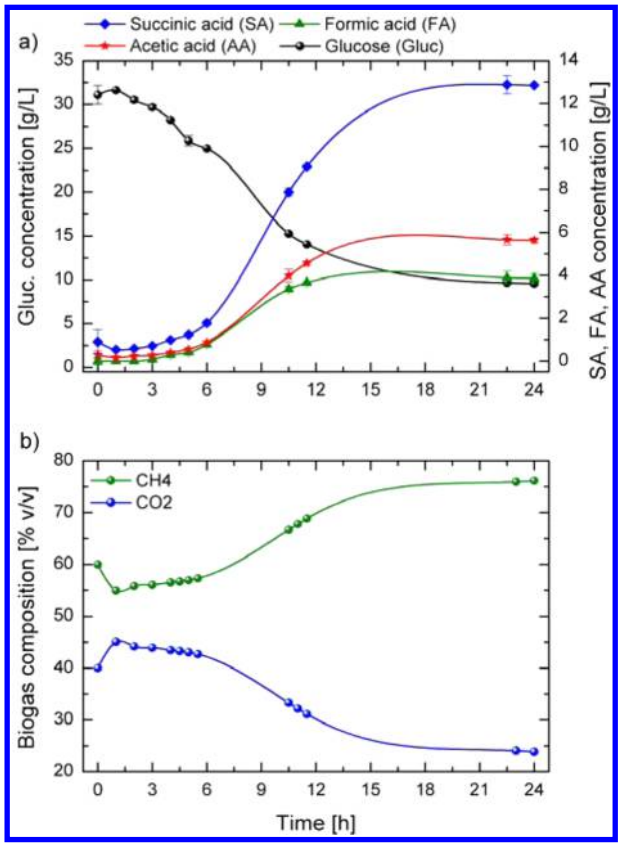

Figure 2. Fermentation (a) and biogas upgrading (b) profiles of *A. succinogenes* at 101.325 kPa and gas−liquid ratio of 8.3:1. Error bars represent the standard deviation of duplicates.

Table 1. System Performance at Different CO_2 Partial Pressures and Gas−Liquid Ratios

	CO_2 partial pressure (kPa)					
	biogas (40% CO_2/60% CH_4)				CO_2 gas	
	40	56	40	56	101.325	140
CO_2 solubility (mM)	9.15	16.7	9.15	16.7	23.16	31.97
gas−liquid ratio	8.3:1	8.3:1	5:1	5:1		
CO_2 fixation rate (L CO_2 L^{-1} d^{-1})	1.35	2.59	1.52	1.77		
final CH_4 content in biogas (% v v^{-1})	76.4	91.1	85.2	95.4		
succinic acid yield (g g^{-1})	0.60	0.62	0.56	0.63	0.62	0.69
succinic acid productivity (g L^{-1} h^{-1})	0.53	0.60	0.53	0.56	0.73	0.80
succinic acid titer (g L^{-1} ± SD)	12.85 ± 0.01	14.39 ± 0.09	12.74 ± 0.22	13.53 ± 0.09	17.53 ± 0.22	19.28 ± 0.24
acetic acid titer (g L^{-1} ± SD)	5.64 ± 0.09	5.10 ± 0.04	6.47 ± 0.45	5.61 ± 0.50	6.13 ± 0.14	5.58 ± 0.13
formic acid titer (g L^{-1} ± SD)	3.86 ± 0.22	3.55 ± 0.02	5.16 ± 0.34	4.35 ± 0.48	4.32 ± 0.27	3.93 ± 0.24

knowledge, this is the first study reporting simultaneous biogas upgrading and succinic acid production by means of this novel concept.

Improving Process Performance. It was investigated if increasing pressure in the system (therefore increasing CO_2 solubility) could significantly increase final CH_4 content in biogas. To study the effect of pressure, the gas−liquid ratio of the system was first kept constant (8.3:1). When total pressure was increased to 140 kPa (CO_2 partial pressure of 56 kPa) the final methane content in biogas was increased to fixation, thereby increasing CH_4 purity in biogas and favorably affecting the succinic acid production. Gas−liquid ratio in the system was reduced to 5:1 in order to investigate its effect on the process performance, especially if CH_4 content could be increased further. At the end of fermentation, CH_4 content was increased from 85.2% at 101.325 kPa to 95.4% at 140 kPa (see Table 1). When comparing the overall CO_2 consumption rate during 24 h fermentation, a 16.4% increase (from 1.52 to 1.77 L CO_2 L^{-1} d^{-1}) was achieved. The final succinic acid titer increased by 6.2% ($p < 0.05$) from 101.325 to 140 kPa (see Table 1).

Additionally, there was a positive effect on the succinic acid yield when pressure was increased, improving it by 13.8% (from 0.56 to 0.63 g g^{-1}). Increased pressure appears to favor succinic acid metabolism in relation to other fermentation products. Increase of the CO_2 pressure from 101.325 to 140 kPa resulted in decrease of the final formic acid titer by 15.7% ($p < 0.05$) compared to that at 101.325 kPa. However, in this case the final acetic acid titer was not found to be significantly different. By reaching 95.4% CH_4 content, the system (gas−liquid ratio 5:1) was able to upgrade the biogas (60% CH_4, 40% CO_2) to a purity similar to that of commercial biogas upgrading technologies (95−98% CH_4 content).[5] Although countries have different standards for purity of natural gas used for gas grid injection and vehicle fuel, purity of 95−98% CH_4 is a common target.[27]

System Performance at Different CO_2 Partial Pressures. One of the key factors in succinic acid fermentation is the availability of CO_2.[25,26] Many studies focusing on succinic acid production have increased the CO_2 availability by addition of salts such as $MgCO_3$, $NaHCO_3$, or $CaCO_3$. Through addition of such salts the CO_2 solubility is greatly increased.[25] Nevertheless, from an industrial perspective the addition of these salts will likely increase the cost of the fermentation process and certainly any solid residue left will increase the cost of downstream processing. Many studies have investigated the use of CO_2 gas in succinic acid production up to 101.325 kPa, while few or no studies have investigated the effect of raising the total pressure (> 101.325 kPa) in the system. For this reason, experiments were conducted using CO_2 gas as the only CO_2 source to study the effect of increasing pressure in the system. The first approach was to predict the effect of increasing pressure in the system (from 101.325 to 140 kPa) by means of an empirical model (SI) together with our experimental conditions (pH, temperature, and culture broth composition). Model prediction showed an increase of 38.1% in CO_2 solubility, which was confirmed later by experimental determination (Figure 3).

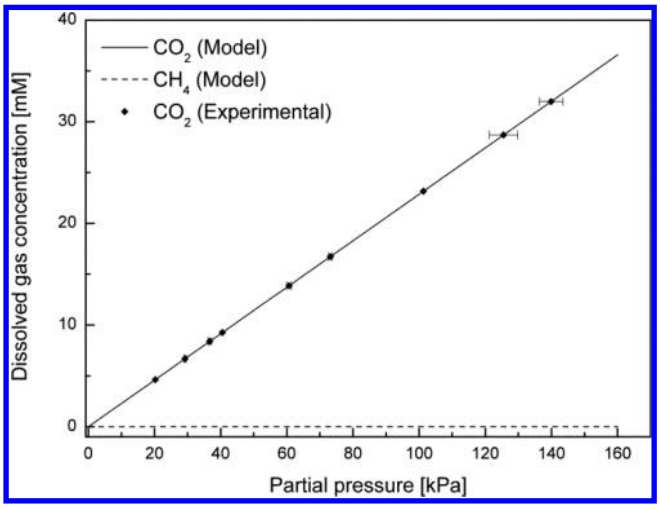

Figure 3. Calculated dissolved CO_2 (—) and CH_4 (- - -) concentrations in culture broth as a function of CO_2 and CH_4 partial pressure using the model and dissolved CO_2 concentration in the culture broth determined experimentally at different CO_2 partial pressures (◆).

As observed in Table 1, the increment of pressure favorably affected the succinic acid production. Comparing the two experimental conditions, the succinic acid titer was observed to be 10.0% higher ($p < 0.05$) at 140 kPa compared to 101.325 kPa. At 140 kPa the succinic acid yield was calculated to be 0.69 g g^{-1}, which represents an increase of 11.5%. Additionally the productivity at 24 h increased from 0.73 to 0.80 g L^{-1} h^{-1}. When comparing results obtained at 101.325 kPa to similar studies, the yield and productivity were 12.7% and 35.4% lower.[26] However, these differences are mainly due to the higher initial glucose concentration used in that study.[26] The final acetic and formic acid titers after 24 h were found not to be significantly different ($p < 0.05$) when comparing both pressure conditions.

In summary, the system described in this work was able to simultaneously upgrade biogas to CH_4 purity of 95.4% and produce succinic acid. To the best of our knowledge this study represents the first investigation of biosuccinic acid production

along with CH_4 purification through utilization of CO_2 fixating bacterium *A. succinogenes* 130Z. By increasing pressure in the system, higher CO_2 solubility was achieved, thereby positively affecting succinic acid yield and titer, CO_2 consumption rate, and CH_4 purity. This work serves as a state-of-the-art study about the integration of production of a biobased bulk chemical and purification of a gaseous energy carrier. Further studies involving the optimization of the system are necessary to fully demonstrate its economic feasibility. Finally, the implementation of this concept on a larger scale may promote its practical application thereby contributing to a worldwide biobased economy.

■ ASSOCIATED CONTENT

Supporting Information

Model description and details, as well as additional fermentation data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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