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*Published in:*  
Environmental Science and Technology

*Link to article, DOI:*  
[10.1021/es504000h](https://doi.org/10.1021/es504000h)

*Publication date:*  
2014

*Document Version*  
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

*Citation (APA):*  
Gunnarsson, I. B., Alvarado-Morales, M., & Angelidaki, I. (2014). Utilization of CO<sub>2</sub> fixating bacterium *Actinobacillus succinogenes* 130Z for simultaneous biogas upgrading and bio-succinic acid production. *Environmental Science and Technology*, 48(20), 12464-12468. DOI: 10.1021/es504000h

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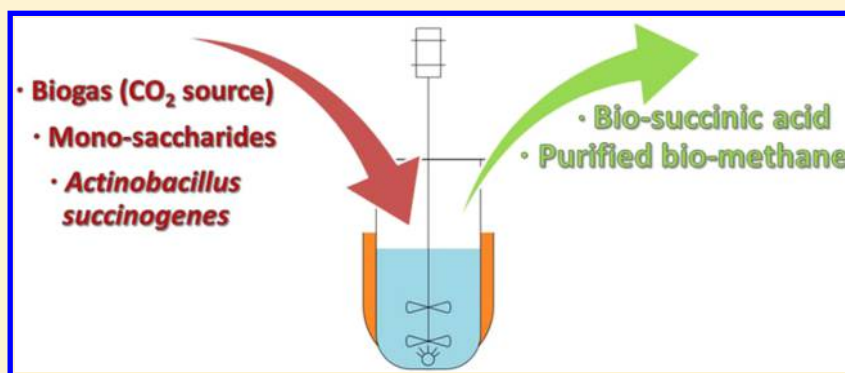
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# Utilization of CO<sub>2</sub> Fixating Bacterium *Actinobacillus succinogenes* 130Z for Simultaneous Biogas Upgrading and Biosuccinic Acid Production

Ingólfur B. Gunnarsson, Merlin Alvarado-Morales, and Irimi Angelidaki\*

Department of Environmental Engineering, Technical University of Denmark, DK-2800 Kongens Lyngby, Denmark

**S** Supporting Information



**ABSTRACT:** Biogas is an attractive renewable energy carrier. However, it contains CO<sub>2</sub> which limits its use for certain applications. Here we report a novel approach for removing CO<sub>2</sub> from biogas and capturing it as a biochemical through a biological process. This approach entails converting CO<sub>2</sub> into biosuccinic acid using the bacterial strain *Actinobacillus succinogenes* 130Z, and simultaneously producing high-purity CH<sub>4</sub> (> 95%). Results showed that when pressure during fermentation was increased from 101.325 to 140 kPa, higher CO<sub>2</sub> solubility was achieved, thereby positively affecting final succinic acid yield and titer, CO<sub>2</sub> consumption rate, and CH<sub>4</sub> purity. When using biogas as the only CO<sub>2</sub> source at 140 kPa, the CO<sub>2</sub> consumption rate corresponded to 2.59 L CO<sub>2</sub> L<sup>-1</sup> d<sup>-1</sup> with a final succinic acid titer of 14.4 g L<sup>-1</sup>. Under this pressure condition, the highest succinic acid yield and biogas quality reached corresponded to 0.635 g g<sup>-1</sup> and 95.4% (v v<sup>-1</sup>) CH<sub>4</sub> 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<sub>2</sub> emissions 15–30% by 2020. With the EU commission trying to implement its emissions trading system (EU ETS),<sup>1</sup> firms are likely to be charged a carbon tax of €10 per metric tonne of CO<sub>2</sub> emissions by the end of 2014 (predicted to reach €13 by the end of 2015).<sup>2</sup> 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.<sup>3</sup> 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.<sup>4</sup> Biogas, produced through anaerobic digestion of organic material, is generally composed of 50–75% CH<sub>4</sub> and 25–50% CO<sub>2</sub>. 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<sub>4</sub> needs to be purified (upgrading).<sup>5</sup> By removing CO<sub>2</sub> 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<sub>2</sub> where it is captured from the biogas (thereby increasing the CH<sub>4</sub> purity) and then released into the atmosphere, contributing to the global warming effect.<sup>5</sup> The necessity for reducing CO<sub>2</sub> emissions has never been greater, as recently the amount of CO<sub>2</sub> in earth's atmosphere was observed to exceed 400 ppm for the first time since measurements started 55 years ago,<sup>6</sup> also the fact that current technologies for CO<sub>2</sub> capturing and storage are costly, therefore processes that use CO<sub>2</sub> as a raw material are likely to become increasingly important.<sup>7</sup> An example of such a process is biological production of succinic acid through bacterial

Received: August 15, 2014

Revised: September 23, 2014

Accepted: October 2, 2014

Published: October 2, 2014

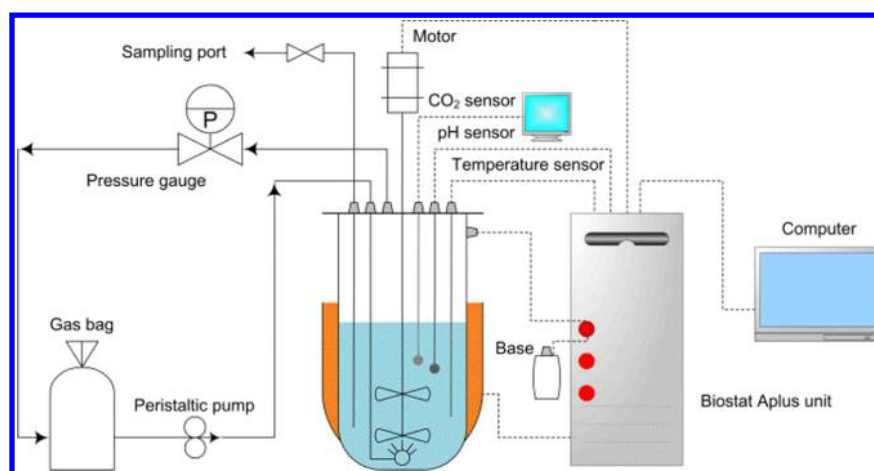


Figure 1. Experimental setup.

fermentation, where CO<sub>2</sub> fixation is performed by *Actinobacillus succinogenes* or any other bacterium capable of producing succinic acid.<sup>8</sup> Succinic acid is a four-carbon diacid with the chemical formula C<sub>4</sub>H<sub>6</sub>O<sub>4</sub> 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)).<sup>8,9</sup> Producing succinic acid biologically through fermentation has the advantage of consuming 1 mol of CO<sub>2</sub> per 1 mol of succinic acid produced, opposed to producing it chemically through hydrogenation of maleic acid as most of it is currently produced.<sup>10</sup> Substantial CO<sub>2</sub> 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<sub>2</sub> emissions.<sup>11</sup> A number of bacterial strains are able to produce succinic acid through fermentation, including *Actinobacillus succinogenes*,<sup>12</sup> *Mannheimia succiniciproducens*,<sup>13</sup> *Anaerobiospirillum succiniciproducens*,<sup>14</sup> *Corynebacterium glutamicum*,<sup>15</sup> and recombinant *Escherichia coli*.<sup>16</sup> *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 ( $\leq 158 \text{ g L}^{-1}$  D-glucose), but also tolerates high levels of succinic acid ( $> 70 \text{ g L}^{-1}$ ).<sup>12,17</sup> Additionally *A. succinogenes* has a broad preference of carbon sources such as glucose, xylose, arabinose, galactose, maltose, fructose, sucrose, cellobiose, lactose, mannitol, arabinol, 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.<sup>18–22</sup> The fermentation end products of *A. succinogenes* are succinic acid as the main metabolite, in addition to formate, acetate, and ethanol.<sup>12,23</sup> The supply of CO<sub>2</sub> is a key factor in the production of succinic acid by *A. succinogenes*. A higher concentration of dissolved CO<sub>2</sub> 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.<sup>24</sup> When using biogas as the only CO<sub>2</sub> source, the maximum dissolved CO<sub>2</sub> concentration in the culture broth corresponds to its solubility at the corresponding CO<sub>2</sub> partial pressure in the biogas (25–50% (v v<sup>-1</sup>)). However, one way to make CO<sub>2</sub> 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<sub>2</sub> in culture broth. The model and parameters used in this work were retrieved from an earlier study unless otherwise stated (see Supporting Information (SI)).<sup>25</sup> In this study we demonstrate a new biogas upgrading technology, which makes use of bacterial fermentation to simultaneously produce high-purity CH<sub>4</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> gets solubilized in the culture broth. As fermentation progresses, CO<sub>2</sub> decreases in the liquid phase (due to consumption by bacterial fermentation) which, according to Henry's law, forces more CO<sub>2</sub> to the gas phase to be dissolved into the culture broth. In this way the CH<sub>4</sub> 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  $-80\text{ }^{\circ}\text{C}$  until used. Seed culture medium was composed of (g/L) glucose (10.0), yeast extract (5.0),  $\text{NaHCO}_3$  (10.0),  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  (9.6), and  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$  (20.3). Medium was sterilized at  $121\text{ }^{\circ}\text{C}$  for 20 min. Seed culture was cultivated at  $37\text{ }^{\circ}\text{C}$  and 150 rpm in 50-mL sealed anaerobic bottles containing 30 mL of medium and inoculated with 1 mL of  $-80\text{ }^{\circ}\text{C}$  glycerol stock culture. Biogas upgrading and succinic acid production batch experiments were conducted in duplicates. Fermentation experiments were conducted using biogas (60%  $\text{CH}_4$ , 40%  $\text{CO}_2$ ) or 100%  $\text{CO}_2$  as only source of  $\text{CO}_2$  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),  $\text{K}_2\text{HPO}_4$  (3.0),  $\text{MgCl}_2$  (0.2),  $\text{CaCl}_2$  (0.2), and  $\text{NaCl}$  (1.0). Medium was sterilized at  $121\text{ }^{\circ}\text{C}$  for 20 min. Sterile glucose solution was added separately to the bioreactors to get a glucose concentration of  $30\text{--}32\text{ g L}^{-1}$ . All batch fermentations in 3-L bioreactors were carried out at  $37\text{ }^{\circ}\text{C}$ , 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.  $\text{NaOH}$  (8 M) was added automatically to maintain the pH at 6.75 during fermentation.  $\text{N}_2$  gas was used in all cases to create anaerobic conditions prior to start of fermentation.

**Analytical Methods.** Dissolved  $\text{CO}_2$  in fermentation broth was determined experimentally using an InPro 5000i (Mettler Toledo, Switzerland)  $\text{CO}_2$  sensor controlled by M400 transmitter (Mettler Toledo, Switzerland). Gas containing 100, 40, and 20% ( $v/v$ )  $\text{CO}_2$  was used to determine the dissolved  $\text{CO}_2$  in fermentation broth at 101.325, 120, and 140 kPa pressure with temperature and pH fixed at  $37\text{ }^{\circ}\text{C}$  and 6.8, respectively. Cell growth was monitored by measuring the optical density at 660 nm ( $\text{OD}_{660}$ ) using a spectrophotometer (Jenway Buch and Holm A/S 64050UV/vis). At  $\text{OD}_{660}$  of 1.0, *A. succinogenes* 130Z has a concentration of  $0.626\text{ g dry cell weight (DCW) L}^{-1}$  (data not 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 ( $300\text{ mm} \times 7.8\text{ mm}$ ) with  $4\text{ mM H}_2\text{SO}_4$  as an eluent at a flow rate of  $0.6\text{ mL min}^{-1}$  with column oven temperature set to  $63.5\text{ }^{\circ}\text{C}$ . 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.,  $\text{CO}_2$ ) in a pure solvent (e.g., water) can be calculated by Henry's law:

$$p_{\text{CO}_2} = H_0 C_{\text{CO}_2}$$

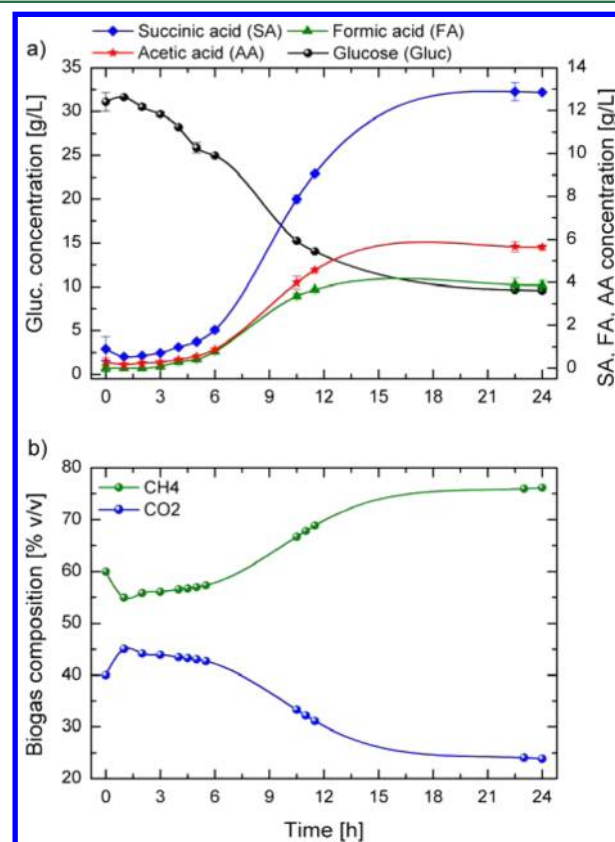
where  $p_{\text{CO}_2}$  is the  $\text{CO}_2$  partial pressure in a gas mixture (kPa),  $H_0$  is the Henry's constant for  $\text{CO}_2$  in a pure solvent ( $\text{kPa L mol}^{-1}$ ), and  $C_{\text{CO}_2}$  is the concentration of dissolved  $\text{CO}_2$  in the liquid phase ( $\text{mol L}^{-1}$ ). However, in a culture broth different kinds of organic substances and salts are always present which affects the

solubility of  $\text{CO}_2$ . The effect of pH, temperature, and the organic substances and salts on  $\text{CO}_2$  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%  $\text{CH}_4$ , 40%  $\text{CO}_2$ ) was used as the only source of  $\text{CO}_2$  for succinic acid fermentation (Figure 2a) using a gas–liquid ratio of 8.3:1. At



**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.

atmospheric pressure ( $\text{CO}_2$  partial pressure of 40 kPa), the maximum solubility of  $\text{CO}_2$  corresponds to  $9.15\text{ mM}$ . As shown in Figure 2b at 101.325 kPa, the final biogas composition at the end of the fermentation corresponded to 76.4%  $\text{CH}_4$ , 24.6%  $\text{CO}_2$ . The  $\text{CO}_2$  partial pressure lessens as  $\text{CO}_2$  is consumed during the course of the fermentation. This resulted in a decrease of the solubility of  $\text{CO}_2$  ( $5.4\text{ mM}$ ) in the culture broth at the end of the fermentation. In terms of succinic acid production, the final titer was  $12.85\text{ g L}^{-1}$  with a yield of  $0.60\text{ g g}^{-1}$ . When fermentation was performed at 101.325 kPa, the overall  $\text{CO}_2$  consumption rate during 24 h fermentation corresponded to  $1.35\text{ L CO}_2\text{ L}^{-1}\text{ d}^{-1}$ . Succinic acid yield and productivity were in agreement with those reported in previous studies.<sup>26</sup> To our

Table 1. System Performance at Different CO<sub>2</sub> Partial Pressures and Gas–Liquid Ratios

	CO <sub>2</sub> partial pressure (kPa)					
	biogas (40% CO <sub>2</sub> /60% CH <sub>4</sub> )				CO <sub>2</sub> gas	
	40	56	40	56	101.325	140
CO <sub>2</sub> 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 <sub>2</sub> fixation rate (L CO <sub>2</sub> L <sup>-1</sup> d <sup>-1</sup> )	1.35	2.59	1.52	1.77		
final CH <sub>4</sub> content in biogas (% v v <sup>-1</sup> )	76.4	91.1	85.2	95.4		
succinic acid yield (g g <sup>-1</sup> )	0.60	0.62	0.56	0.63	0.62	0.69
succinic acid productivity (g L <sup>-1</sup> h <sup>-1</sup> )	0.53	0.60	0.53	0.56	0.73	0.80
succinic acid titer (g L <sup>-1</sup> ± 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 <sup>-1</sup> ± 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 <sup>-1</sup> ± 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<sub>2</sub> solubility) could significantly increase final CH<sub>4</sub> 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<sub>2</sub> partial pressure of 56 kPa) the final methane content in biogas was increased to fixation, thereby increasing CH<sub>4</sub> 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<sub>4</sub> content could be increased further. At the end of fermentation, CH<sub>4</sub> content was increased from 85.2% at 101.325 kPa to 95.4% at 140 kPa (see Table 1). When comparing the overall CO<sub>2</sub> consumption rate during 24 h fermentation, a 16.4% increase (from 1.52 to 1.77 L CO<sub>2</sub> L<sup>-1</sup> d<sup>-1</sup>) 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<sup>-1</sup>). Increased pressure appears to favor succinic acid metabolism in relation to other fermentation products. Increase of the CO<sub>2</sub> 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<sub>4</sub> content, the system (gas–liquid ratio 5:1) was able to upgrade the biogas (60% CH<sub>4</sub>, 40% CO<sub>2</sub>) to a purity similar to that of commercial biogas upgrading technologies (95–98% CH<sub>4</sub> content).<sup>5</sup> Although countries have different standards for purity of natural gas used for gas grid injection and vehicle fuel, purity of 95–98% CH<sub>4</sub> is a common target.<sup>27</sup>

**System Performance at Different CO<sub>2</sub> Partial Pressures.** One of the key factors in succinic acid fermentation is the availability of CO<sub>2</sub>.<sup>25,26</sup> Many studies focusing on succinic acid production have increased the CO<sub>2</sub> availability by addition of salts such as MgCO<sub>3</sub>, NaHCO<sub>3</sub>, or CaCO<sub>3</sub>. Through addition of such salts the CO<sub>2</sub> solubility is greatly increased.<sup>25</sup> 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<sub>2</sub> 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<sub>2</sub> gas as the only CO<sub>2</sub> 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<sub>2</sub> solubility, which was confirmed later by experimental determination (Figure 3).

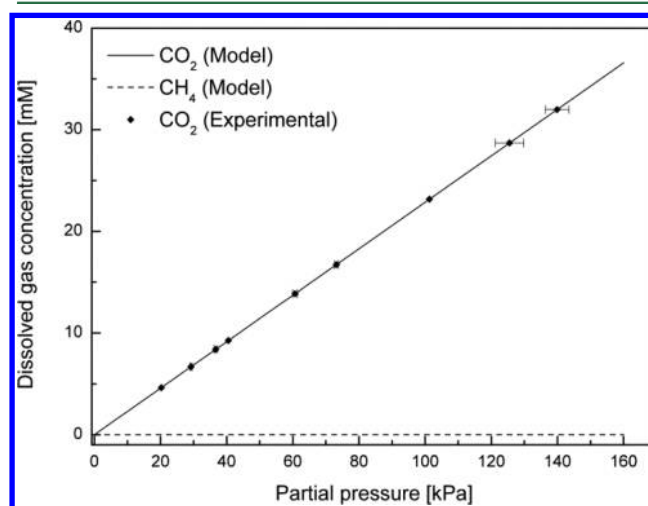


Figure 3. Calculated dissolved CO<sub>2</sub> (—) and CH<sub>4</sub> (---) concentrations in culture broth as a function of CO<sub>2</sub> and CH<sub>4</sub> partial pressure using the model and dissolved CO<sub>2</sub> concentration in the culture broth determined experimentally at different CO<sub>2</sub> 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<sup>-1</sup>, which represents an increase of 11.5%. Additionally the productivity at 24 h increased from 0.73 to 0.80 g L<sup>-1</sup> h<sup>-1</sup>. When comparing results obtained at 101.325 kPa to similar studies, the yield and productivity were 12.7% and 35.4% lower.<sup>26</sup> However, these differences are mainly due to the higher initial glucose concentration used in that study.<sup>26</sup> 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<sub>4</sub> 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<sub>4</sub> purification through utilization of CO<sub>2</sub> fixing bacterium *A. succinogenes* 130Z. By increasing pressure in the system, higher CO<sub>2</sub> solubility was achieved, thereby positively affecting succinic acid yield and titer, CO<sub>2</sub> consumption rate, and CH<sub>4</sub> 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>.

## ■ AUTHOR INFORMATION

### Corresponding Author

\*Phone: +45 45251429; e-mail: [iria@env.dtu.dk](mailto:iria@env.dtu.dk).

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

This study is the result of work done in the Biorefinery Øresund project. We thank Interreg IVA for the financial support given.

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