Succinate production from CO2-grown microalgal biomass as carbon source using engineered Corynebacterium glutamicum through consolidated bioprocessing

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The potential for production of chemicals from microalgal biomass has been considered as an alternative route for CO2 mitigation and establishment of biorefineries. This study presents the development of consolidated bioprocessing for succinate production from microalgal biomass using engineered Corynebacterium glutamicum. Starch-degrading and succinate-producing C. glutamicum strains produced succinate (0.16 g succinate/g total carbon source) from a mixture of starch and glucose as a model microalgal biomass. Subsequently, the engineered C. glutamicum strains were able to produce succinate (0.28 g succinate/g of total sugars including starch) from pretreated microalgal biomass of CO2-grown Chlamydomonas reinhardtii. For the first time, this work shows succinate production from CO2 via sequential fermentations of CO2-grown microalgae and engineered C. glutamicum. Therefore, consolidated bioprocessing based on microalgal biomass could be useful to promote variety of biorefineries.

Metabolic engineering, aiming for enhanced production of desired bio-products through modification of cellular metabolism, has enabled to construct microbial cell factories including engineered Escherichia coli and yeast for production of native or non-native biochemical from fermentable sugars1. However, efficient conversion of lignocellulosic biomass to fermentable sugars is critical for production of biofuels and chemicals in industrial scales2. The recalcitrant structures of lignocellulose hamper its efficient degradation into simple sugars3. Although various methods have been developed to break the structures of lignin and crystalline cellulose prior to enzymatic hydrolysis4, the pretreatment step is a still bottleneck for fermentation of lignocellulosic biomass. On the other hand, microalgal cultivation as a potential platform for production of biofuels or chemicals has several positive aspects5, including high productivity per-acre over lignocellulosic feedstock resources6,7. In addition, microalgal lipids could be converted to biodiesel, and other components of microalgal biomass including carbohydrates, polyunsaturated fatty acid and proteins can serve as CO2-derived carbon sources for biorefineries i.e. direct conversion of Spirulina to ethanol without pretreatment or enzymatic hydrolysis8. Thus, we have considered microalgal biomass as a potential feedstock to replace the lignocellulosic biomass and to produce value-added chemicals.

Corynebacterium glutamicum is a predominantly aerobic, non-pathogenic, biotin-auxotrophic Gram-positive bacterium. It is used industrially for amino acid production, in particular the flavor enhancer L-glutamate and the feed additive L-lysine9. Recently, engineering of amino acid-producing C. glutamicum have been enabled to utilize hydrolysates of rice straw, wheat bran, and molasses10. Moreover, recent studies have indicated the potential of C. glutamicum as a microbial cell factory to produce other commercially relevant chemicals such as succinate, isobutanol, cadaverine, and ethanol11–13. To broaden the substrate range, metabolic engineering of C. glutamicum
has been employed to utilize non-native carbon sources such as cellobiose, N-acetylglucosamine, or starch. Particularly, starch was used as sole carbon source for production of cadaverin, L-glutamate, L-lysine and organic acids in C. glutamicum via either enzyme secretion or surface-display of α-amylases. In this report, we focused on highly accumulated starch in microalgae biomass as potential carbon source for C. glutamicum. Here, we engineered a succinate-producing C. glutamicum strain to secrete starch-degrading α-amylases to produce succinate from CO2-derived microalgal biomass, as an example of consolidated bioprocessing for microalgal biomass (Fig. 1).

Results
Utilization of soluble starch by engineered C. glutamicum. Carbohydrates including starch are major constituents in the microalgal biomass of Chlamydomonas reinhardtii UTEX 90 when the algae were starved for other essential elements such as nitrogen. As C. glutamicum wild type ATCC 13032 is unable to utilize starch, we first implemented this ability by constructing strains Cg-pSbAmyA and Cg-pBlAmyS capable of secreting α-amylase into the medium (Table 1). C. glutamicum wild type harboring the empty plasmid pBbEB1c did not consume soluble starch (Sigma; no glucose detected) at all and showed no growth (Fig. 2). Strain Cg-pSbAmyA did not completely consume 0.5% (w/v) soluble starch (Sigma) within 56 hr and reached a maximal cell dry weight (cdw) of 0.5 ± 0.01 g/L. However, strain Cg-pBlAmyS completely consumed 0.5% soluble starch (Sigma) in 6 hr and reached a maximal biomass of 1.23 g ± 0.01 cdw/L, which was the same biomass of the Cg-pBbEB1c grown on 0.5% (w/v) glucose as sole carbon source (1.23 g ± 0.01 cdw/L). Thus, we measured α-amylase volume activity in the supernatants. In comparisons to Cg-pSbAmyA culture medium, 2-fold increased activities of Cg-pBlAmyS culture medium were measured at 8 hr when Cg-pBlAmyS cells reached almost the maximum cell growth (Table 2). However, low amylase activities (less than 100 U/L) of Cg-pSbAmyA were not enough to completely utilize the starch as sole carbon source. Nonetheless, we successfully constructed starch-degrading C. glutamicum strains via secreting the α-amylase and applied this system for succinate production.

Succinate production from soluble starch by engineered C. glutamicum. The succinate-producing C. glutamicum strain BL-1, which carries deletions of the genes pqo (encoding for pyruvate:menaquinone oxidoreductase), pta-ackA, sdhCAB, and cat, did not consume soluble starch (Sigma; no glucose detected) at all and showed no growth (Fig. 2). Strain Cg-pSbAmyA did not completely consume 0.5% (w/v) soluble starch (Sigma) within 56 hr and reached a maximal cell dry weight (cdw) of 0.5 ± 0.01 g/L. However, strain Cg-pBlAmyS completely consumed 0.5% soluble starch (Sigma) in 6 hr and reached a maximal biomass of 1.23 g ± 0.01 cdw/L, which was the same biomass of the Cg-pBbEB1c grown on 0.5% (w/v) glucose as sole carbon source (1.23 g ± 0.01 cdw/L). Thus, we measured α-amylase volume activity in the supernatants. In comparisons to Cg-pSbAmyA culture medium, 2-fold increased activities of Cg-pBlAmyS culture medium were measured at 8 hr when Cg-pBlAmyS cells reached almost the maximum cell growth (Table 2). However, low amylase activities (less than 100 U/L) of Cg-pSbAmyA were not enough to completely utilize the starch as sole carbon source. Nonetheless, we successfully constructed starch-degrading C. glutamicum strains via secreting the α-amylase and applied this system for succinate production.

Table 1 | Bacteria strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics | Source or reference |
|-------------------|--------------------------|---------------------|
| **Strains**       |                          |                     |
| E. coli HIT-DH5x   | F' (80d lacZ M15) [lacZYA-argF] U169 hsdR17(r m-1) recA1 endA1 relA1 deoR96 | RBC Bioscience     |
| C. glutamicum ATCC 13032 | Wild type | ATCC                |
| Cg-pBbEB1c        | Wild type harboring pBbEB1c | This study          |
| Cg-pSbAmyA        | Wild type harboring pBbEB1c-torA-SbAmyA(cg.co) | This study          |
| Cg-pBlAmyS        | Wild type harboring pBbEB1c-torA-BlAmyS(cg.co) | This study          |
| BL-1              | C. glutamicum ATCC 13032 derivative with in-frame deletions of pqq, pta-ackA, sdhCAB, and cat | 12                  |
| BL-1-pBbEB1c      | BL-1 harboring pBbEB1c | This study          |
| BL-1-pSbAmyA      | BL-1 harboring pBbEB1c-torA-SbAmyA(cg.co) | This study          |
| BL-1-pBlAmyS      | BL-1 harboring pBbEB1c-torA-BlAmyS(cg.co) | This study          |
| C. reinhardtii    | Wild type | UTEX90              |
| **Plasmids**      |                          |                     |
| pBbEB1c           | CoE1 [Ec], pBL1 [Cg], Cm’, P pervasive. BglBrick sites, CoryneBrick vector | 29                  |
| pBbEB1c-torA-SbAmyA(cg.co) | pBbEB1c derivative containing the codon-optimized S. bovis amyA gene with a TorA signal peptide | This study          |
| pBbEB1c-torA-BlAmyS(cg.co) | pBbEB1c derivative containing the codon-optimized B. licheniformis amyS gene carrying the mutations Q268S and N265Y and a TorA signal peptide | This study          |
Figure 2 | Profile of soluble starch and the growth of *C. glutamicum* strains. *Cg-pBbEB1c* as a control strain (A) and α-amylase-secreting *Cg-pBbAmyA* (B) and *Cg-pBlAmyS* (C) strains were cultivated with 0.5% (w/v) soluble starch (Sigma) as a sole carbon source. Optical density was measured at 600 nm (closed circle; black). Soluble starch (closed square; blue) in the supernatant was quantified. Mean values and standard deviations of duplicate cultures are shown (s.d. less than 1% not shown).

### Table 2 | Secreted α-amylase volume activities (U/L) in the culture medium

| Time | Carbon sources | Starch-degrading *C. glutamicum* wild type derivatives | Starch-degrading and succinate-producing *C. glutamicum* BL-1 derivatives |
|------|----------------|-----------------------------------------------------|---------------------------------------------------------------|
|      |                | *Cg-pSlAmyS*                                         | *BL-1-pBbAmyS*                                               |
| 8 hr | Starch*        | 90.8 ± 4.4                                          | n.m.                                                         |
| 12 hr| Glc + Starch**| 560.3 ± 25.3                                        | 737.2 ± 16.4                                                |
|      | Starch         | 99.3 ± 1.3                                          | n.m.                                                         |
| 24 hr| Glc + Starch   | 598.3 ± 19.6                                        | 729.4 ± 15.1                                                |
|      | Starch         | 76.5 ± 5.6                                          | n.m.                                                         |
|      | Glc + Starch   | 589.3 ± 6.9                                         | 839.1 ± 24.6                                                |

Note: One unit (U) of activity was defined as the amount of enzyme required to release 1 μmol of CNP from N3-G5-μCNP per minute at 37°C. Mean values and standard deviations of duplicate cultures are shown.

*5% starch was used as sole carbon source.

**A mixture of 0.5% (w/v) glucose and 0.5% (w/v) starch was used as sole carbon source. Since the BL-1-pBbAmyA and BL-1-pBlAmyS were only cultivated with a mixture of glucose and starch, enzyme activity data is not measured (n.m.).
glutamicum strains and produced succinate from soluble starch using the engineered strains.

CO2-derived succinate production from microalgal biomass by engineered C. glutamicum. Finally, we applied our engineered strains to utilize CO2-derived microalgal biomass and to produce CO2-derived succinate. To obtain the microalgal biomass, C. reinhardtii UTEX 90 was grown photoautotrophically with 5% (v/v) CO2 and 95% (v/v) air bubbling. Disrupted microalgal biomass was centrifuged and the resulting supernatant was used as only carbon source for succinate production in GgXII medium. It contained 0.2% total sugars, of which 50% were determined to be soluble starch, similar to previous work. Compared with BL-1-pBbEB1c (0.56 g ± 0.01 cdw/L), BL-1-pSbAmyA and BL-1-pBlAmyS showed doubled biomass formation (1.05 ± 0.01 g cdw/L and 1.02 ± 0.01 g cdw/L) in 24 hr, respectively (Fig. 4).

As shown for the cell culture on a mixture of starch and glucose in this study, the BL-1-pSbAmyA and BL-1-pBlAmyS also degraded soluble starch in microalgal biomass from the initial cell growth. Then, the rest of sugars were slowly consumed, but 10% of initial total sugars were not utilized at all, which could be pentose sugars not utilized by the strains, such as xylose or arabinose.

The strains BL-1-pBbAmyS and BL-1-pSbAmyA produced 0.49 ± 0.01 g/L and 0.50 ± 0.01 g/L succinate after 24 hr from 0.2% total sugar including 0.1% starch in pretreated microalgal biomass, respectively. The control strain BL-1-pBbEB1c unable to utilize starch produced only 30% of the succinate (0.15 g/L ± 0.001) found for the amylase-secreting strains. Moreover, the yields (succinate/total sugars used) of BL-1-pBlAmyS (0.28 g/g) and BL-1-pSbAmyA (0.28 g/g) were significantly higher compared to the BL-1-pBbEB1c strain (0.20 g/g) when pretreated microalgal biomass was used. Finally, for the first time, we successfully produced succinate from CO2-derived microalgal biomass using engineered strains.
strains capable of degrading starch without a need for additional enzyme treatment.

**Discussion**

Microbial biomass of *C. reinhardtii* is a remarkable carbohydrate feedstock to provide the carbon sources for microbial fermentations. Often separate hydrolysis and fermentation (SHF) or simultaneous biomass to provide the carbon sources for microbial fermentations. Discussion strains capable of degrading starch without a need for additional targets. Also, application of cell display system20 (i.e. the (50 mL in 250 mL baffled Erlenmeyer flasks) containing either 0.5% soluble starch when appropriate. For the utilization of soluble starch or microalgal biomass and correlation factor of 0.25 g cell dry weight per liter for OD600 is able of producing enzyme for saccharification and producing the enzyme activity measurement. For the determination of α-amylase volume activity (U/L), the supernatant of the cells were collected and the samples were analyzed using an α-amylase measurement kit (Kikkoman, Tokyo, Japan) using 2-chloro-4-nitrophenyl-α-D-glucopyranoside (N3-G5-β-CNP) as the substrate in the previous studies23. The assay mixture was incubated at 37 °C for 10 min, and the enzymatic reaction was terminated by adding 800 μL of a reaction stop solution, α-Amylase activity was determined according to the manufacturer’s instruction by measuring the absorbance of the liberated 2-chloro-4-nitrophenol (CNP) at 400 nm. One unit (U) of activity was defined as the amount of enzyme required to release 1 μmol of CNP from N3-G5-β-CNP per minute at 37 °C. Determination of starch, total sugar, and succinate. For quantification of starch10, different dilutions of the culture supernatant were assayed with Lugol solution containing iodine (1.5 g/L) and potassium iodide (15 g/L), leading to the formation of a blue complex that was measured in a spectrophotometer at 530 nm. For quantification of total sugars, a colorimetric method based on the phenol–sulfuric acid reaction was used to determine the amount of total sugars including the starch in pretreated microalgal biomass. Succinate in the supernatant was quantified by HPLC as described previously21. **Enzyme activity measurement.** For the determination of α-amylase volume activity (U/L), the supernatant of the cells were collected and the samples were analyzed using an α-amylase measurement kit (Kikkoman, Tokyo, Japan) using 2-chloro-4-nitrophenyl-α-D-glucopyranoside (N3-G5-β-CNP) as the substrate in the previous studies23. The assay mixture was incubated at 37 °C for 10 min, and the enzymatic reaction was terminated by adding 800 μL of a reaction stop solution, α-Amylase activity was determined according to the manufacturer’s instruction by measuring the absorbance of the liberated 2-chloro-4-nitrophenol (CNP) at 400 nm. One unit (U) of activity was defined as the amount of enzyme required to release 1 μmol of CNP from N3-G5-β-CNP per minute at 37 °C. Determination of starch, total sugar, and succinate. For quantification of starch10, different dilutions of the culture supernatant were assayed with Lugol solution containing iodine (1.5 g/L) and potassium iodide (15 g/L), leading to the formation of a blue complex that was measured in a spectrophotometer at 530 nm. For quantification of total sugars, a colorimetric method based on the phenol–sulfuric acid reaction was used to determine the amount of total sugars including the starch in pretreated microalgal biomass. Succinate in the supernatant was quantified by HPLC as described previously21. **Determination of starch, total sugar, and succinate.** For quantification of starch10, different dilutions of the culture supernatant were assayed with Lugol solution containing iodine (1.5 g/L) and potassium iodide (15 g/L), leading to the formation of a blue complex that was measured in a spectrophotometer at 530 nm. For quantification of total sugars, a colorimetric method based on the phenol–sulfuric acid reaction was used to determine the amount of total sugars including the starch in pretreated microalgal biomass. Succinate in the supernatant was quantified by HPLC as described previously21. **Methods**

**Bacterial strains and growth conditions.** Strains used in this study are listed in Table 1. For cloning purposes *E. coli* DH5α was used and grown in lysogeny broth medium (LB) when appropriate, the medium was supplemented with 25 μg/mL chloramphenicol. *C. glutamicum* ATCC 13032 and its derivatives were cultivated in BHIS medium** at 30 °C and 200 rpm and 7.5 μg/mL chloramphenicol was added when appropriate. For the utilization of soluble starch or microbial biomass and succinate production, engineered *C. glutamicum* were pre-cultivated in the BHIS medium overnight and then incubated aerobically in the CxGII defined medium (50 mL in 250 mL baffled Erlenmeyer flasks) containing either 0.5% soluble starch (Sigma-Aldrich) or pretreated microbial biomass as sole carbon source** at 30 °C on a rotary shaker at 200 rpm with 7.5 μg/mL chloramphenicol. The biomass concentration was calculated from OD<sub>600</sub> values using an experimental determined correlation factor of 0.25 g cell dry weight per liter for OD<sub>600</sub> = 1.0.

Microbial cultivation for biomass preparation. To obtain a large amount of algal biomass containing starch for succinate production by *C. glutamicum*, a freshwater green alga, *C. reinhardtii* UTEX 89, was grown phototrophically in Tris-acetate-phosphate medium** without acetic acid (TAP-C). Cultivations were carried out at 23 °C in 20 L of the TAP-C medium in a 25 L photobioreactor with 65 mL/min of 5% (v/v) CO<sub>2</sub> and 95% (v/v) air aeration and 100 μM/l/s of illumination with a dark/ light cycle (12:12 hr). The cells were harvested by centrifugation at 5,000 × g for 10 min after two weeks cultivation including nitrogen starvation (1.15 g cell dry weight/L). The lyophilized cells were suspended in distilled water, disrupted by glass bead-beating, and centrifuged (10 min at 5,000 × g). The supernatant was used as only carbon source for the cultivation of *C. glutamicum*. Construction of α-amylase-secreting *C. glutamicum*. The amyA (NCBI no. AB000829.1) and amyS (NCBI M38579.1) genes from *Streptococcus bovis* and *Bacillus licheniformis*, respectively, were chosen since they were well characterized and studied in *C. glutamicum* and *E. coli*22. Each target gene was synthesized (GenScript, USA) with codon-optimization for *C. glutamicum* (represented as cgc.co). Each gene was assembled using a standard BglBrick cloning method, where the target gene is inserted at the EcoRI and XhoI sites of the CorrynBleck plasmid p8B8EC22. The Tat-specific TorA signal peptide sequence from *C. glutamicum* was added to the coding sequence of the target gene where the native signal sequences was already deleted. The plasmids used in this study are listed in Table 1. For the transformation of *C. glutamicum*, competent cell preparation and electroporation were performed with the plasmids according to a previously described protocol23 with some modifications. 1. Keasling, J. D. From yeast to alkaloids. Nat. Chem. Biol. 4, 524–525 (2008). 2. Bokinsky, G. et al. Synthesis of three advanced biofuels from ionic liquid-pretreated switchgrass using engineered *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 108, 19949–19954 (2011). 3. Vajee-Kolstad, G. et al. An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. Science 330, 219–222 (2010). 4. Taylor, M. P., Mulako, I., Tuflin, M. & Cowan, D. Understanding physiological responses to pre-treatment inhibitors in ethanologenic fermentations. Biotechnol. J. 7, 1169–1181 (2012). 5. Parmar, A., Singh, N. K., Pandey, A., Gnansounou, E. & Madamwar, D. Cyanobacteria and microalgae: a positive prospect for biofuels. Bioresour. Technol. 102, 10163–10172 (2011). 6. Yen, H.-W., Hu, I.-C., Chen, C.-Y., Ho, S.-H., Lee, D.-J. & Chang, J.-S. Microalgae-based biorefinery - From biofuels to natural products. Bioresour. Technol. 135, 166–174 (2012). 7. John, R. P., Anisha, G. S., Nampoothiri, K. M. & Pandey, A. Micro and macroalgae biomass: a renewable source for biofuel. Bioresour. Technol. 102, 186–193 (2011). 8. Aikawa, S. et al. Direct conversion of Spirulina to ethanol without pretreatment or enzymatic hydrolysis processes. Energy Environ. Sci. 6, 1844–1849 (2013). 9. Kalinowski, J. et al. The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of l-aspartate-derived amino acids and vitamin. *J. Biotechnol.* 104, 5–25 (2003). 10. Xu, J., Zhang, J., Guo, Y., Zai, Y. & Zhang, W. Improvement of cell growth and L-lysine production by genetically modified *Corynebacterium glutamicum* during growth on molasses. J. Ind. Microbiol. Biotechnol. 40, 1423–1432 (2013). 11. Becker, J. & Wittmann, C. Bio-based production of chemicals, materials and fuels –*Corynebacterium glutamicum* as versatile cell factory. Curr. Opin. Biotechnol. 23, 631–640 (2012). 12. Litsanov, B., Kabus, A., Brocker, M. & Rott, M. Efficient aerobic succinate production from glucose in minimal medium with *Corynebacterium glutamicum*. Microb. Biotechnol. 5, 116–128 (2012). 13. Wu, H.-M. & Park, J. B. Bioinformatics in development of synthetic biology platforms and metabolic engineering of *Corynebacterium glutamicum*. *J. Biotechnol.* 140, 43–51 (2014). 14. Uhde, A. et al. Glucosamine as carbon source for amino acid-producing *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. 97, 1679–1687 (2013).
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Author contributions

H.M.W. conceived and supervised the project, J.L. and H.M.W. designed experiments. J.L. and H.M.W. performed experiments. Y.U., S.J.S., M.B. and M.-K.O. oversaw the project. J.L., M.B. and H.M.W. wrote and revised the manuscript. All authors analyzed data and discussed the results and reviewed the manuscript.

Additional information

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