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Succinate production from CO_2-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 CO_2 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 CO_2-grown *Chlamydomonas reinhardtii*. For the first time, this work shows succinate production from CO_2 via sequential fermentations of CO_2-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 sugars[1]. However, efficient conversion of lignocellulosic biomass to fermentable sugars is critical for production of biofuels and chemicals in industrial scales[2].

The recalcitrant structures of lignocellulose hamper its efficient degradation into simple sugars[3]. Although various methods have been developed to break the structures of lignin and crystalline cellulose prior to enzymatic hydrolysis[4], 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 aspects[5], including high productivity per-acre over lignocellulosic feedstock resources[6,7]. 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 CO_2-derived carbon sources for biorefineries i.e. direct conversion of *Spirulina* to ethanol without pretreatment or enzymatic hydrolysis[8]. 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-lysine[9]. Recently, engineering of amino acid-producing *C. glutamicum* have been enabled to utilize hydrolysates of rice straw, wheat bran, and molasses[10]. 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 ethanol[11–13]. To broaden the substrate range, metabolic engineering of *C. glutamicum*

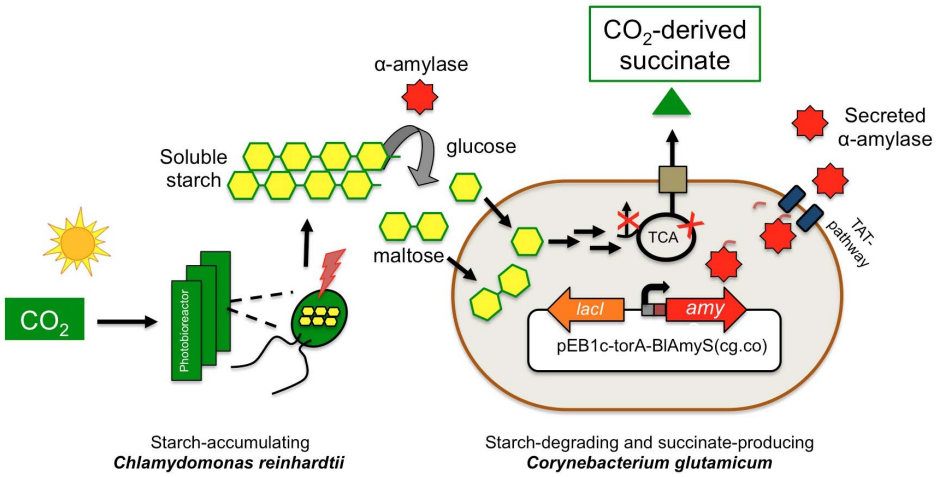

Figure 1 | Scheme of CO_2-derived succinate production from microalgal biomass using engineered *C. glutamicum* through consolidated bioprocessing. Engineered *C. glutamicum* secrets α-amylase to degrade soluble starch derived from extracts of CO_2-grown *C. reinhardtii* and produces succinate from total sugars from microalgal biomass without enzyme addition.

has been employed to utilize non-native carbon sources such as cellobiose, N-acetylglucosamine, or starch[14–16]. Particularly, starch was used as sole carbon source for production of cadaverin[17], L-glutamate[18], L-lysine[19] and organic acids[20] 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 CO_2-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[12], which carries deletions of the genes *pqo* (encoding for pyruvate:menaquinone oxidoreductase), *pta-ackA* (encoding for

Table 1 | Bacteria strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli HIT-DH5α	F⁻(80d *lacZ* M15) (*lacZYA-argF*) U169 *hsdR17*(r^- m^+) *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 *pqo, 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	ColE1 (Ec), pBL1 (Cg), Cm^r, P_{trc}, 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

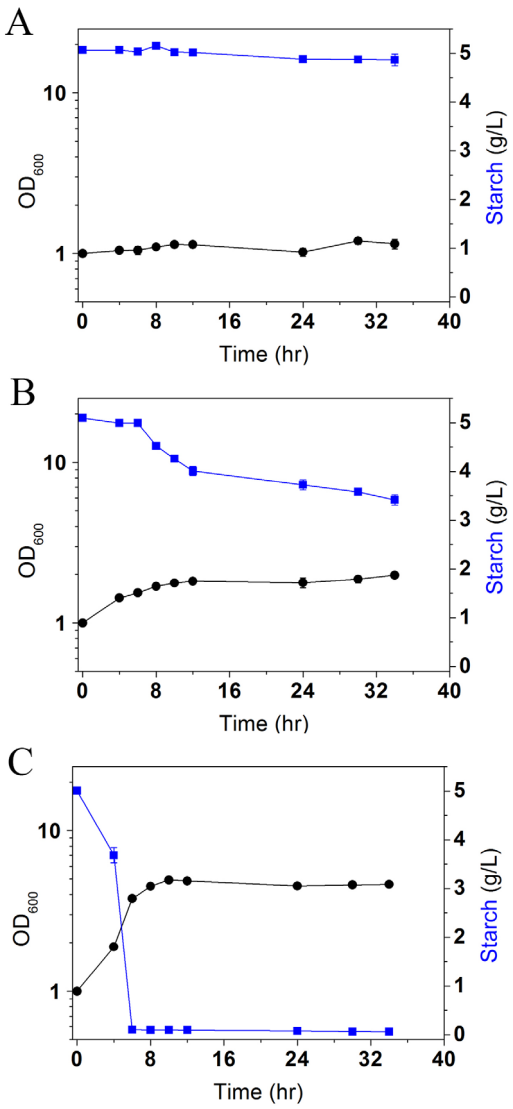

Figure 2 | Profile of soluble starch and the growth of *C. glutamicum* strains. *Cg-pBbEB1c* as a control strain (A) and α-amylase-secreting *Cg-pSbAmyA* (B) and *Cg-pBlAmyS* (C) strains were cultivated with 0.5% (w/v) soluble starch (Sigma) as a sole carbon source. Optical density was measured at 600 nm (closed circle; black). Soluble starch (closed square; blue) in the supernatant was quantified. Mean values and standard deviations of triplicate cultures are shown (s.d. less than 1% not shown).

phosphate acetyltransferase and aceate kinase), *sdhCAB* (encoding for succinate dehydrogenase complex), and *cat* (encoding for acetyl-CoA:CoA transferase) was transformed with the plasmids pBbEB1c-torA-*SbAmyA*(cg.co) and pBbEB1c-torA-*BlAmyS*(cg.co). As a result, we constructed BL-1-*pSbAmyA* and BL-1-*pBlAmyS* strains.

Microalgal biomass consist mainly carbohydrates (60%, based on cdw), protein (8.3%, based on cdw) and others[21]. 58% of carbohydrates in microalgal biomass were soluble starch. We used a mixture of 0.5% (w/v) glucose and 0.5% (w/v) soluble starch as the model carbon source of microalgal biomass for succinate production. These strains and a control strain carrying the vector without an amylase gene (BL-1-*pBbEB1c*) were tested for their ability to utilize a model carbon source of microalgal biomass. 0.5% glucose was supplemented as an additional carbon source to the CgXII defined medium with 0.5% soluble starch. The control strain BL-1-*pBbEB1c* in a mixture of glucose and starch grew (maximal growth 1.78 g ± 0.01 cdw/L) where 0.5% glucose was completely depleted and 0.5% starch was not consumed at all. On the other hand, the strains BL-1-*pBlAmyS* and BL-1-*pSbAmyA* showed an almost doubled biomass formation (2.64 g ± 0.01 cdw/L and 2.89 ± 0.17 g cdw/L), respectively, in comparison to the control strain (Fig. 3). The BL-1-*pSbAmyA* and BL-1-*pBlAmyS* degraded soluble starch in the early stage of cell growth. Then, both BL-1-*pBlAmyS* and BL-1-*pSbAmyA* strains completely consumed both glucose and starch within 16 hr.

When cultivated with the mixture of 0.5% glucose and 0.5% of starch, strains BL-1-*pSbAmyA* and BL-1-*pBlAmyS* produced 1.56 ± 0.01 g/L succinate after 24 hr and 1.44 ± 0.01 g/L succinate after 14 hr, respectively. The yields (g/g) (succinate/total sugars; assuming 100% conversion of starch to glucose) of BL-1-*pBlAmyS* (0.16 g/g) and BL-1-*pSbAmyA* (0.14 g/g) were slightly higher compared to the BL-1 strain cultivated on glucose (0.12 g/g)[12]. As a result, strain BL-1-*pBlAmyS* showed the fastest cell growth and succinate production due to fast utilization of both glucose and starch. When glucose was supplemented to starch-minimal medium as microalgal biomass, the amylase volume activities of *Cg-pSbAmyA* or *Cg-pBlAmyS* were significantly increased by 6.2-folds or 3.1-fold, respectively, compared to the strains with starch as sole carbon source (Table 2) and consequently, the starches were rapidly consumed after 4 hr (lag phase). Initial supply of glucose is necessary for the *C. glutamicum*-secreting SbAmyA or BlAmyS strains that increase the amylase volume activities and efficiently hydrolyze soluble starch. The increased volume activities of SbAmyA or BlAmyS were also shown in *BL-1-pSbAmyA* and *BL-1-pBlAmyS*, which is important for efficient consolidated bioprocessing of microalgal biomass where initial fermentable carbohydrates exist (Fig. 4). For the first time, we successfully constructed starch-degrading and succinate-producing *C.*

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

		Secreted α-amylase volume activities (U/L)			
		Starch-degrading *C. glutamicum* wild type derivatives		Starch-degrading and succinate-producing *C. glutamicum* BL-1 derivatives	
Time	Carbon sources	*Cg-pSbAmyA*	*Cg-pBlAmyS*	BL-1-*pSbAmyA*	BL-1-*pBlAmyS*
8 hr	Starch*	90.8 ± 4.4	203.1 ± 3.8	n.m.	n.m.
	Glc + Starch**	560.3 ± 25.3	643.1 ± 25.3	737.5 ± 16.4	748.7 ± 10.7
12 hr	Starch	99.3 ± 1.3	217.0 ± 8.2	n.m.	n.m.
	Glc + Starch	598.3 ± 19.6	657.4 ± 12.0	729.4 ± 15.1	806.8 ± 17.0
24 hr	Starch	76.5 ± 5.6	205.8 ± 20.2	n.m.	n.m.
	Glc + Starch	589.3 ± 6.9	307.9 ± 20.0	839.1 ± 24.6	665.9 ± 60.7

Note: 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. Mean values and standard deviations of duplicate cultures are shown.
*0.5% 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-*pSbAmyA* and BL-1-*pSlAmyS* were only cultivated with a mixture of glucose and starch, enzyme activity data is not measured (n.m.).

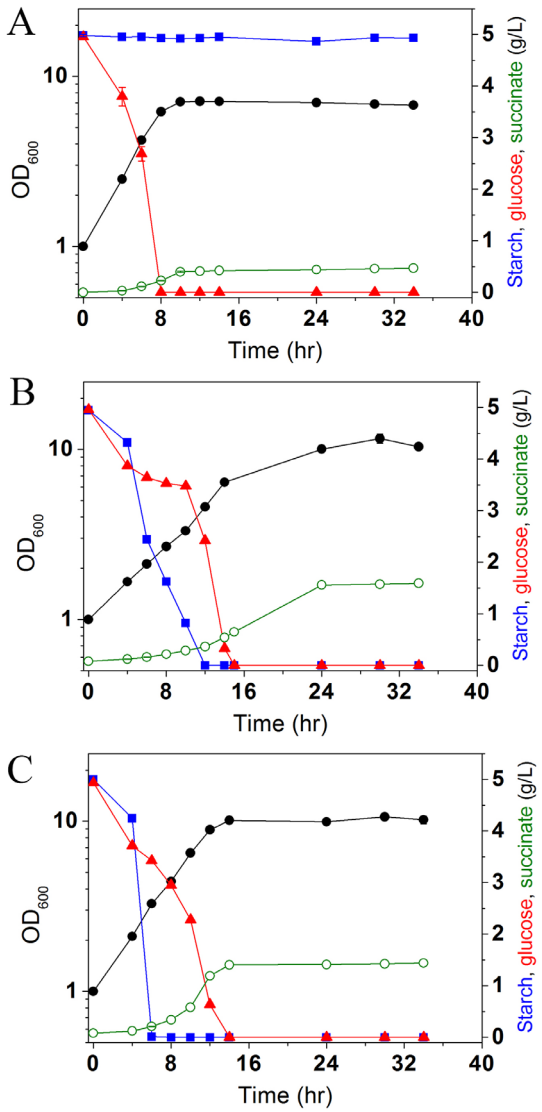

Figure 3 | Profile of glucose, soluble starch, succinate, and the growth of *C. glutamicum* strains. The succinate-producing *C. glutamicum* strains BL-1-*pBbEB1c* (A), BL-1-*pSbAmyA* (B), and BL-1-*pBlAmyS* (C) were cultivated with a mixture of 0.5% (w/v) soluble starch and 0.5% (w/v) glucose as an model microalgal biomass. Optical density was measured at 600 nm (closed circle; black). Soluble starch (closed square; blue), glucose (closed triangle, red), and succinate (open circle, green) in the supernatant were quantified. Mean values and standard deviations of triplicate cultures are shown (s.d. less than 1% not shown).

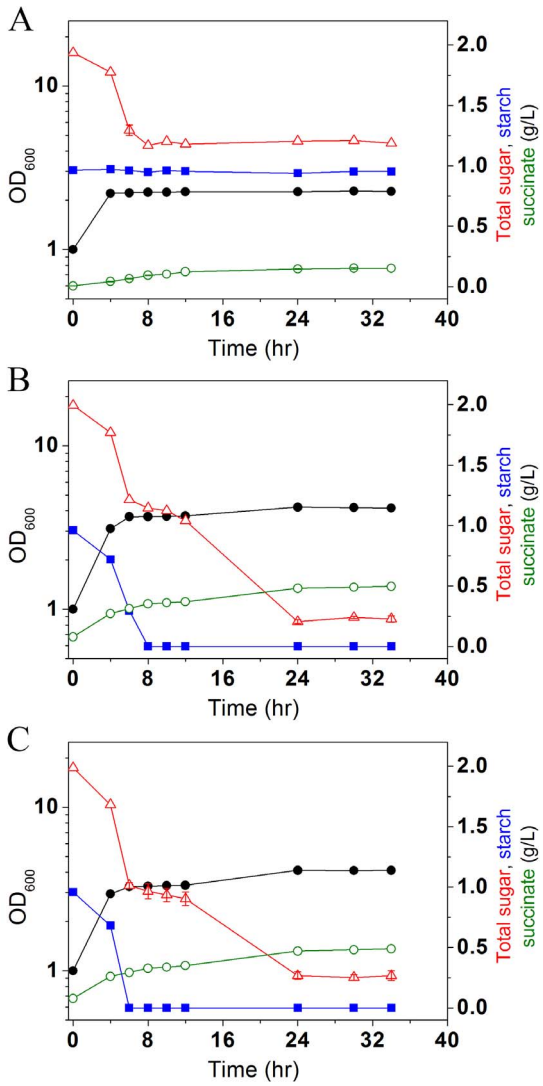

Figure 4 | Profile of total sugar, soluble starch, succinate, and the growth of *C. glutamicum* strains. Succinate-producing *C. glutamicum* strain BL-1-*pBbEB1c* (A), BL-1-*pSbAmyA* (B), and BL-1-*pBlAmyS* (C) were cultivated with pretreated microalgal biomass as sole carbon source. Total sugar already contains soluble starch in this experiment. Optical density was measured at 600 nm (closed circle; black). Total sugar (open triangle; red), soluble starch (closed square; blue), and succinate (open circle; green) in the supernatant were quantified. Mean values and standard deviations of triplicate cultures are shown (s.d. less than 1% not shown).

glutamicum strains and produced succinate from soluble starch using the engineered strains.

CO_2-derived succinate production from microalgal biomass by engineered *C. glutamicum*. Finally, we applied our engineered strains to utilize CO_2-derived microalgal biomass and to produce CO_2-derived succinate. To obtain the microalgal biomass, *C. reinhardtii* UTEX 90 was grown photoautrophically with 5% (v/v) CO_2 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 CgXII medium. It contained 0.2% total sugars, of which 50% were determined to be soluble starch, similar to previous work[21]. 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-*pBlAmyS* 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 CO_2-derived microalgal biomass using engineered

strains capable of degrading starch without a need for additional enzyme treatment.

Discussion

Microalgal 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 saccharification and fermentation (SSF) process have been applied for production for the production of bioethanol[22,23]. However, additional enzyme loading at either SHF or SSF process could be a crucial bottleneck for economically feasible bioprocess to produce value-added chemicals or biofuels[24]. Thus, consolidated bioprocessing was suggested as an alternative strategy that microbial strain is capable of producing enzyme for saccharification and producing the target chemicals such as biofuels from lignocellulosic biomass[25,26]. In this study, we suggested another type of consolidated bioprocessing based on microalgal biomass. A succinate-producing *C. glutamicum* strain was capable of degrading starch by secreting α-amylase and successfully fermented microalgal biomass and produce succinate without amylase additions.

BL-1-*pBlAmyS* (0.28 g/g) strain and its fermentation of showed remarkable yield of succinate production due to the utilization of soluble starch that *C. glutamicum* wild type is not able to consume. This consolidated bioprocessing based on microalgal biomass with the best strain BL-1-*pBlAmyS* does not require additional costs for loading enzymes but produce the high yield of succinate, compared to the succinate producer BL-1. Furthermore, efficient hydrolysis of soluble starch and co-uptake of other carbohydrates and their cooperative sugar metabolisms could be useful to ensure faster cell growth of *C. glutamicum* and higher production of succinate. Metabolic engineering by optimizing gene expression of AmyS from *B. licheniformis* could be possible by tuning translation strengths on ribosomal binding site or changing different signal peptides. Additional sugar transporters and hydrolytic enzymes could be necessary to uptake unused carbohydrates in the total sugars because 10% of total sugars in microalgal biomass were not fermentable. In addition to extensive studies on sugar metabolism[27], pentose-sugar fermentations of engineered *C. glutamicum* have been well investigated[28,29]. Current synthetic platform (CoryneBrick[29]) for the gene expression in this study can be easily expanded for additional gene expression of targets. Also, application of cell display system[20] (i.e. the *B. subtilis* PgsA and *C. glutamicum* PorC protein as anchor) of target amylases in *C. glutamicum* could be alternative to increase hydrolysis of soluble starch and production of succinate.

Microalgal biomass was shown to serve as an efficient carbon source for the microbial production of succinate, which is considered as a platform chemical, when suitably engineered strains were used which are capable of starch degradation by the secretion of amylases. Ultimately, consolidated bioprocessing based on microalgal biomass offers another options to resolve issues of alternative energy resources, global warming, human health and food security.

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[30] at 30°C and 200 rpm and 7.5 μg/mL chloramphenicol was added when appropriate. For the utilization of soluble starch or microalgal biomass and succinate production, engineered *C. glutamicum* were pre-cultivated in the BHIS medium overnight and then incubated aerobically in the CgXII defined medium (50 mL in 250 mL baffled Erlenmeyer flasks) containing either 0.5% soluble starch (Sigma-Aldrich) or pretreated microalgal biomass as sole carbon source[30] at 30°C on a rotary shaker at 200 rpm with 7.5 μg/mL chloramphenicol. The biomass concentration was calculated from OD_{600} values using an experimental determined correlation factor of 0.25 g cell dry weight per liter for $OD_{600} = 1$[31].

Microalgal 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 90, was grown photoautotrophically in Tris-acetate-phosphate medium[21] 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_2 and 95% (v/v) air aeration and 100 μE/m²/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 resuspended 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 no. M38570.1) genes from *Streptococcus bovis* and *Bacillus licheniformis*, respectively, were chosen since they were well characterized and studied in *C. glutamicum*[19] and *E. coli*[32]. Each target gene was synthesized (Genscript, USA) with codon-optimization for *C. glutamicum* (represented as cg.co). Each gene was assembled using a standard BglBrick cloning method, where the target gene is inserted at the *Eco*RI and *Xho*I sites of the CoryneBrick plasmid pBbEB1c[29]. The Tat-specific TorA signal peptide sequence from *C. glutamicum*[33] 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 protocol[34] with some modifications.

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-nitrohenyl 6^5-azido-6^5-deoxy- β-maltopentaoside (N3-G5-β-CNP) as the substrate in the previous studies[35]. 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 starch[16], different dilutions of the culture supernatant were assayed with Lugols 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[36], 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 previously[12].

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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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