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Supplementary Materials

Fermentation of pigment-extracted microalgal residue using yeast cell-surface display: Direct high-density ethanol production with competitive life cycle impacts

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▪ Methods and materials

Determination of biomass concentration, nitrate concentration, and CO_2 fixation rate

Briefly, microalgal cells were harvested by centrifugation at 6688 rpm for 1 min at room temperature, washed twice with de-ionized water and then lyophilized to obtain the dry cell weight (DCW). The biomass concentration was calculated according to the formula defined in Equation (S1). Nitrate concentration was determined according to a previously reported method [1]. Briefly, the culture samples were filtered through a 0.22 μm filter and diluted with de-ionized water. The absorbance was measured at 220 nm via UV/Vis spectrophotometry. The CO_2 fixation rate (F_{CO_2}) was calculated according to the formulas defined in Equations (S2).

$$1.0\ OD_{685} = 0.7\text{-}0.8\ g\ DCW/L \qquad (S1)$$

$$F_{CO_2}\ (mg/L/d) = \frac{1.88 \times \text{Biomass concentration (g/L)}}{\text{Cultivation day (d)}} \times 1000 \qquad (S2)$$

Life cycle assessment (LCA)

The assumptions of four alternatives used for LCA are as follows: (1) the evaluation of LCA was ranged from “microalgae biomass” to “ethanol production or other value-added products”. The process of microalgal cultivation was negligible; (2) the optimal parameters and processes involved in production of 1 g ethanol were selected for further analysis of environmental impacts and economic output of four alternatives. For alternative-1, 0.005% α-amylase (90℃) and 0.2% glucoamylase (55℃) were the optimum conditions used for liquefaction and saccharification in separate hydrolysis and fermentation (SHF) process, respectively. However, the optimum conditions of acid pretreatment for ethanol production in alternative-2 was: 1% H_2SO_4, initial algal biomass concentration of 50 g/L, and 12-h fermentation with SHF process. The alternative-3 achieved the largest ethanol yield with the addition of 1 g/L lysozyme, using a recombinant strain MT8-1δGS to operate fermentation process for 48 h. In this study, 300 g JSC4 residue fermented

72 h reached the optimum ethanol production in the presence of 50 g/L amylase- and cellulase-displayed recombinant yeasts.

- **Figures:**

(a) 10 L photobioreactor used for strain JSC4 culture

(b) Cell growth and CO_2 fixation of strain JSC4

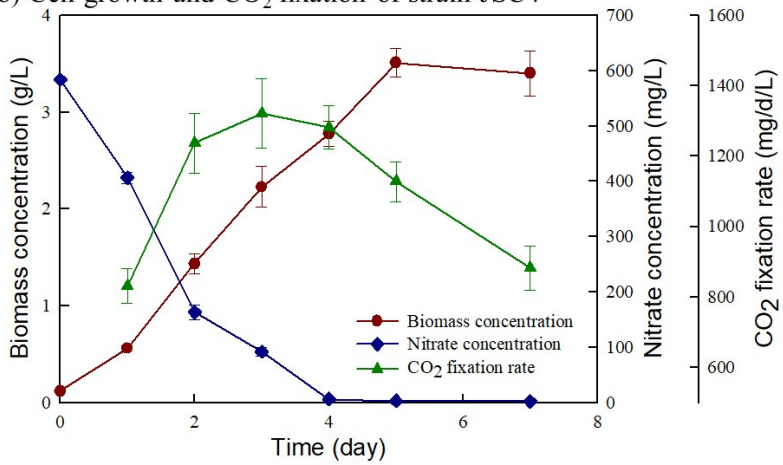

Fig. S1 Time-course profiles of (b) biomass concentration, nitrogen concentration, and CO_2 fixation during the growth of *Chlamydomonas* sp. JSC4 in 10 L PBR (a). Error bars indicate the standard deviation of three replicates. (Other conditions: light source, TL5 lamp; light intensity = 400 μmol/m^2/s; CO_2 aeration = 2%; CO_2 flow rate = 0.05 vvm)

▪ Tables

Table S1 Yeast strains used for displaying cellulases or amylases in this study

Strains	Description	Source
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Life Technologies
EG-D-CBHI-D-CBHII-D	EG-D-CBHI-D (pDI9-CBHII$_D$), display of BGL, EG, CBHI, and CBHII	This study
BY-AASS/GASS/GASS	BY-AASS/GASS/pIU5GA-SS, display of α-amylase and glucoamylase	This study

BGL, β-glucosidase; EG, endoglucanase; CBHI, cellobiohydrolase II; CBHII, cellobiohydrolase II.

Table S2 Characteristics of integrative plasmids used in this study.

[a] Integrative vector without display cassette

Plasmids	Description	Source
pRDH227	*Hyg*, expression of *Chrysosporium lucknowense CBHII* gene	This study
pDI9-CBH1$_D$	*MET15*, display of *T. emersonii* CBHI	This study
pDI9-CBH2$_D$	*MET15*, display of *C. lucknowense* CBHII	This study
pRS403	*HIS3*[a]	Agilent Technologies
pRS406	*URA3*[a]	Agilent Technologies
pIUPGSBAAG	*URA3*, display of *Streptococcus bovis* α-amylase	[2]
pδU-PGGlucRAG	*URA3,* display of *Rhizopus oryze* glucoamylase (δ-Integrative vector)	[3]
pIAA-SS[b]	*HIS3,* display of *S. bovis* α-amylase	This study
pIGA-SS[b]	*HIS3,* display of *R.oryza* glucoamylase	This study
pIU5GA-SS[b]	*URA3*, display of *R.oryza* glucoamylase	This study

[b] The vector fragment containing *SED1* promoter, *SAG1* terminator, and sequence for *SED1*-anchoring region were amplified pIEG-SS [4].

Table S3 PCR primers used for display cellulases in this study

Primers	Sequence
I9a-M-F	ATTAATGAATCGGCCAACGCTGGATATGACTGTGTTGTTGCTGATA
I9a-O-R	GGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGG
O-I9a-F	AAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCC
O-I9b-R	GCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTC
I9b-O-F	AAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACC
I9b-C1-R	TTTTCACCGTCATCACCGAAGGGCCCATGGCTAGGTGT
C1-M-F	ACCTACTTTCTCTCACAAGTCGGATCTATGCGGTGTGAAATAC
C1-I9b-R	CAACAACACAGTCATATCCAGCGTTGGCCGATTCATTA
C2-F	AATACGTTCGCTCTATTAAGATGGCCAAGAAGTTGTTCATTACC
C2-R	GTTGATAATTTACTCGAGCCGAATGGTGGATTTGCGTTCGTTAAC
D-C2-F	CGAACGCAAATCCACCATTCGGCTCGAGTAAATTATCAACTGTCC
P-C2-R	ATGAACAACTTCTTGGCCATCTTAATAGAGCGAACGTATTTT
I9-F	AAGAAGAAATCCGTGCTTACACATT
I9-R	GCTATCCCATGCAAAGATTGTCAACG

Table S4 PCR primers used for display amylases in this study

Primers	Sequence
AA-F	AATACGTTCGCTCTATTAAGATGAGATTTCCTTCAATTTTTACTGC
AA-R	AATAGGACAGTTGATAATTTCTTGTCATCGTCATCCTTGTAGTC
SSvector-F	ACAAGGATGACGATGACAAGAAATTATCAACTGTCCTATTATCTGCC
SSvector-R	AAAATTGAAGGAAATCTCATCTTAATAGAGCGAACGTATTTTATTTTG
GA-F	AATACGTTCGCTCTATTAAGATGCAACTGTTCAATTTGCC
GA-R	GTTGATAATTTACTCGAGCCAGCGGCAGGTGCACCAGCCTTAG
SSvector-F2	AGGCTGGTGCACCTGCCGCTGGCTCGAGTAAATTATCAACTGTCC
SSvector-R2	GGCAAATTGAACAGTTGCATCTTAATAGAGCGAACGTATTTT
I5-F	TGTACTGAGAGTGCACCATATTGTTGTGTAAATGTTCTATCTGACACT
I5-R	ATTGAATTGAAAAGCTGTGGCAGGTTGTGCTCACTGTATATAGTCTC
URA3-F	TATACAGTGAGCACAACCTGCCACAGCTTTTCAATTCAATTCATC
URA3-R	TTTCACACCGCATAGATCCGGGGTAATAACTGATATAATTAAATTGAAG
GASSvector-F2	AATTATATCAGTTATTACCCCGGATCTATGCGGTGTGAAATAC
GASSvector-R2	ATAGAACATTTACACAACAATATGGTGCACTCTCAGTACAATCTG

Table S5 Detailed inventory data of all the alternatives during the process of producing 1 g ethanol

		Alternative-1	Alternative-2	Alternative-3	Alternative-4
Raw materials (g)	Algal biomass	4.25532	4.29185	2.85714	0.41152
	Sulfuric acid	0.42128	1.57946	---	---
	Yeast	0.08511	0.08584 (bacterium)	7.6856	0.68737/1.029
	Yeast extract	4.25532	0.8584	1.54283	0.54743
	Peptone	8.51064	--	5.7142	1.09486
	Glucose	4.25532	1.7168	5.7142	1.09486
	Others	α-amylase: 0.00426 Glucoamylase: 0.17021	KH_2PO_4: 0.17168 $(NH_4)_2SO_4$: 0.08584 $MgSO_4$: 0.04292 $CaCO_3$: 0.8069	Lysozyme: 0.02058 Na_2EDTA: 0.51428	Acetone: 3.229 Additional products: Lutein (2 mg)
Utilities (kW·h)	Centrifuge	0.29787	0.8584	0.17143	0.008232
	Shaker (twice)	5.10638/12.2553	1.54512/3.09024	8.22845/4.11422	1.18541/0.88906
	Others	Water bath: 6.38298	Autoclave: 0.51504	---	Sonication: 0.08232
	Total electricity	24.043	6.009	12.514	2.239

Table S6 Comparison of biomass production, carbohydrate content, and carbohydrate productivity of *Chlamydomonas* sp. JSC4 with other microalgae reported in previous literatures.

Strains	Cultivation time (d)	Cultivation scale and apparatus	Biomass concentration (g/L)[a]	Carbohydrate content (%)	Carbohydrate productivity (mg/L/d)	References
Chlamydomonas sp. JSC4	7	10 L/Photobioreactor (PBR)	3.5	65[b]	438[c]	This study
Scenedesmus obliquus CNW-N	12	1 L/PBR	2.63	30-40	n.d.	[5]
Scenedesmus obliquus AS-F-7-1	12	1 L/PBR	1.13	n.d.	n.d.	[5]
Chlamydomonas vulgaris	6	250 mL/Erlenmeyer flasks	1.70	44	112	[6]
Chlamydomonas reinhradtii	3	4 L/Flat-vertical PBR	1.45	53.1	257	[7]
Chlorella vulgaris (CCAP 211/11B)	14	2 L/Tank bioreactor	0.52	55.0	21	[8]

n.d.: not determined.

[a] maximum of biomass concentration during the cultivation.

[b] maximum of carbohydrate content during the cultivation.

[c] maximum of carbohydrate productivity during the cultivation.

Table S7 Time-course profiles of biochemical composition during the growth of *Chlamydomonas* sp. JSC4.

Content (%)	Cultivation day (d)				
	2	3	4	5	7
Lipid	9.20±0.89	11.91±0.67	15.26±0.07	18.17±0.84	21.78±1.24
Carbohydrate	33.16±0.92	51.07±2.85	58.63±3.51	64.30±1.26	64.65±0.74
Protein	49.98±0.86	29.67±0.28	18.64±0.48	10.19±0.33	6.28±0.10
Others	7.66±0.94	7.35±3.63	7.47±3.13	7.34±2.43	7.29±1.80

Table S8 Detailed inventory data of (a) ecosystem quality, (b) human health, and (c) resources depletion for all alternatives

(a)	Ecosystem quality									
	Agricultural land occupation	Climate change	Freshwater ecotoxicity	Freshwater eutrophication	Marine ecotoxicity	Natural land transformation	Terrestrial acidification	Terrestrial ecotoxicity	Urban land occupation	**Total**
Alternative-1	5.20×10^{-5}	1.10	4.88×10^{-4}	1.68×10^{-3}	1.63×10^{-1}	4.94×10^{-1}	2.54×10^{-3}	8.78×10^{-4}	1.24×10^{-2}	**1.77**
Alternative-2	1.30×10^{-5}	0.275	1.22×10^{-4}	4.20×10^{-4}	4.08×10^{-2}	1.23×10^{-1}	6.33×10^{-4}	2.20×10^{-4}	3.11×10^{-3}	**0.443**
Alternative-3	2.72×10^{-5}	0.573	2.54×10^{-4}	8.75×10^{-4}	8.50×10^{-2}	2.57×10^{-1}	1.32×10^{-3}	4.62×10^{-4}	6.48×10^{-3}	**0.924**
Alternative-4	4.98×10^{-6}	0.103	4.56×10^{-5}	1.57×10^{-4}	1.52×10^{-2}	4.60×10^{-2}	2.37×10^{-4}	8.39×10^{-5}	1.16×10^{-3}	**0.166**

(b)	Human health						
	Climate change	Human toxicity	Ionising radiation	Ozone depletion	Particulate matter formation	Photochemical oxidant formation	**Total**
Alternative-1	1.38	5.24	4.38×10^{-5}	5.75×10^{-6}	9.00×10^{-2}	5.69×10^{-4}	**6.71**
Alternative-2	0.345	1.31	1.10×10^{-5}	1.44×10^{-6}	2.25×10^{-2}	1.42×10^{-4}	**1.68**
Alternative-3	0.720	2.73	2.29×10^{-5}	3.01×10^{-6}	4.69×10^{-2}	2.97×10^{-4}	**3.49**
Alternative-4	0.129	0.498	4.19×10^{-6}	5.47×10^{-7}	8.42×10^{-3}	5.76×10^{-5}	**0.626**

(c)	Resources depletion			**Total scores**
	Resources - fossil depletion	Resources - metal depletion	**Total**	**(Ecosystem quality +Human health +Resources depletion)**
Alternative-1	1.49	1.41×10^{-2}	**1.50**	**9.99**
Alternative-2	0.372	3.58×10^{-3}	**0.375**	**2.50**
Alternative-3	7.75	7.37×10^{-3}	**0.783**	**5.20**
Alternative-4	1.49	1.34×10^{-3}	**0.141**	**0.933**

Table S9 The impact scores of elements on human toxicity and categories of emission to environment

Elements	Category	Impact scores (point)			
		Alternative 1	Alternative 2	Alternative 3	Alternative 4
Selenium (Se)	Emission to water / ground water, long-term	2.298	0.574	1.196	0.214
Manganese (Mg)	Emission to water / ground water, long-term	1.572	0.392	0.818	0.146
Barium (Ba)	Emission to water / ground water, long-term	0.401	0.100	0.209	0.037
Arsenic (As, ion)	Emission to water / ground water, long-term	0.258	0.064	0.134	0.024
Selenium (Se)	Emission to air / low population density	0.218	0.054	0.114	0.020
Molybdenum (Mo)	Emission to water / ground water, long-term	0.193	0.048	0.100	0.018
Selenium (Se)	Emission to water / surface water	0.082	0.020	0.043	0.008
Arsenic (As, ion)	Emission to water / surface water	0.072	0.018	0.038	0.007
Total impacts		**5.238**	**1.310**	**2.726**	**0.489**

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