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Enzymes produced by biomass-degrading bacteria can efficiently hydrolyze algal cell walls and facilitate lipid extraction

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ABSTRACT

The toughness of microalgal cell walls makes lipid extraction and large-scale biodiesel production difficult. This study investigated the enzymatic hydrolysis of algal cell walls, in which the enzymes were produced by eight biomass-degrading bacterial strains. The bacteria were first cultured in mineral salt medium containing 5% (w/v) wheat bran and various lignocellulolytic enzymes, including carboxymethyl cellulase (CMCase), filter paper activity (FPase), xylanase, and laccase were monitored in order to obtain an enzymatic extract. All the strains showed marked CMCase activity, with a range of 3.0–6.9 U ml^{-1} after incubation for 2–5 d. Some strains also produced FPase, xylanase, and laccase. The enzymatic extract was directly added to fresh algae culture at a ratio of 1:3 (v/v) for 48 h. All the bacterial enzymatic extracts significantly disrupted algal cell walls, according to the enhancement of reducing sugar content in the culture. The lipid extraction yield was markedly increased by 10.4–43.9%, depending on the bacteria strains used. Due to its high reducing sugar production and lipid extraction efficiency, *Bacillus* sp. K1 was selected for a time-course experiment. Maximum lipid yield was obtained after 24 h of incubation at the room temperature, with about 40% of the cells were disrupted. These results showed that enzymes produced by biomass-degrading bacteria can weaken and disrupt cell walls and components of algae and facilitate the release of lipids from algae.

1. Introduction

The shortage of petrochemical fuel resources and environmental concerns about global warming has seen a surge in recent years in the search for renewable and sustainable fuels [1]. Biodiesel has been put forward as a viable alternative to fossil fuels because of its environmental benefits, high oxygen ratio, and exceptional combustion characteristics [2,3]. Traditional biodiesel is mainly produced by rapeseed, corn, soybean, oil palm, sunflower, and Jatropha [4,5]. However, such raw materials utilize valuable farmland and therefore compete with food crops [6]. Thus, they have limited value as a long-term global energy supply.

Microalgae are regarded as a very promising feedstock for biodiesel production owing to their high photosynthesis efficiency, short life cycle, and high lipid accumulation [7,8]. Biodiesel obtained from microalgae usually involves the following steps: cultivation, harvesting, lipid extraction, and conversion. Various aspects of the processes of cultivation and harvesting have been well studied [9–11]. Efficient lipid extraction remains an obstacle in biodiesel production, with methods needed to improve the lipid yield from algae [12]. Most algal cell walls are composed of trilayered structures of cellulose, hemicellulose, and trilaminar layers of algaenan and glycoproteins, which are three times stronger than the cell walls of plant cells [13–15]. To release more lipids before lipid extraction, cell disruption is particularly important [16]. The cells can be disrupted via mechanical, physical, chemical, and enzymatic processes [17]. A variety of pretreatment methods, such as bead-beating [18], high-pressure [19], sonication [16], osmotic shock [20], detergent [21], acids [22], and commercial enzymes [23], are available to enhance lipid extraction from algae. However, high energy consumption, high costs of chemicals or potential environmental risks make these methods difficult to scale

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up [17,24].

Some bacteria that secrete lignocellulolytic enzymes, such as cellulase, xylanase, and laccase, have shown tremendous potential to degrade the cell walls [25]. Pretreatment by incubating biomass with bacteria does not involve expensive equipment or hazardous chemicals and is regarded as an economical, eco-friendly, and esthetically acceptable technology to overcome biomass recalcitrance [25,26]. A previous study reported that ligninolytic enzymes secreted by *Anthracophyllum discolor* promoted the attack of different cell wall components of algae and finally weakened these walls [14]. The lipid concentration of *Chlorella vulgaris* treated with 5.0 mg L^{-1} cellulase was 4.8 times higher than that of controls [27]. Cho et al. [23] found that the lipid extraction yield of *Chlorella vulgaris* increased 1.29–1.73-fold, depending on the solvents used, after hydrolysis by cellulases and β-glucosidase as compared to when no enzymatic hydrolysis process was used.

Recently, lipid extraction from wet algal biomass was proposed as a relatively economical technology because it does not require further concentrating and drying of algal biomass [28]. However, the pre-treatment of wet algae by using enzymes produced by biomass-degrading bacteria is still scarce. In this study, the hydrolysis of microalgal cell walls by lignocellulolytic enzymes from eight bacteria strains with high production of these enzymes was investigated. The enzymes, including carboxymethyl cellulase (CMCase), filter paper activity (FPase), xylanase, and laccase, produced by these bacteria were directly added to fresh algal culture to weaken the microalgal cell walls. The hydrolysis ability was then evaluated by lipid extraction from wet microalgae using solvents.

2. Materials and methods

2.1. Chemical and reagents

Carboxymethyl cellulose (CMC), beechwood xylan, 3,5-dinitrosalicylic acid, and 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Filter paper (Whatman No.1) was obtained from ThermoFisher Scientific (San Jose, CA, USA). All other chemicals and reagents were of analytical grade.

2.2. Bacterial and algal strains and culture conditions

The following eight bacterial isolates were used in this study: *Bacillus* sp. A0, *Bacillus* sp. A4, *Exiguobacterium* sp. AS2B, *Pseudomonas* sp. CDS3, *Bacillus* sp. CH2OS1, *Bacillus* sp. K1, *Raoultella* sp. X1, and *Bacillus subtilis* X4. The first six strains have been reported previously by our lab members (Table 1). The last two strains were isolated from forest soil (Thunder Bay, Ontario, Canada) and identified as *Raoultella* sp. X1 and *Bacillus subtilis* X4 (Fig. S1). The strains and their characterized activities are described in Table 1. All the strains were stored at −70 °C in a freezer in our laboratory. Prior to the experiments, all the strains were activated in Luria-Bertani (LB) medium at 37 °C, with agitation at 200 rpm for 12 h. The bacteria were then cultured on a large scale in mineral salt medium (0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, and 0.02% peptone) containing 5% (w/v) wheat bran biomass for 6 d. The production of lignocellulolytic enzymes, including CMCase, FPase, xylanase, and laccase, was monitored every day. The culture medium from different strains with the highest CMCase activity was separated by centrifuging at 12,000*g* for 3 min. The supernatant was then collected and passed through 0.22-μm filters for degradation experiments.

The algal strain *Chlorella zofingiensis* was obtained from Dr. Lu of Algaen Corporation, Winston Salem, U.S [29]. The cells were grown in BG-11 medium [30] at room temperature (25 ± 1 °C) under continuous photon flux at a density of 100 ± 2 μmol m^{-2} s^{-1} under cool white fluorescent light illumination, with light/dark cycles of 16:8 h and with constant shaking at 150 rpm. After 3 wk of incubation, the algae culture was used to evaluate the degradation ability of the bacterial strains.

2.3. Screening and evaluation of cellulase enzymatic activity

To evaluate the algal degradation ability of the bacterial strains, the strains were detected on an agar plates using algae biomass as the sole carbon source according to the method of Kasana et al. [31]. Briefly, 5 μl of overnight-grown culture was inoculated on agar plates containing 900 ml of algae cell pellets. The cell pellets were collected by centrifuging at 8000*g* for 10 min and washed three times using distilled water (equal to 5 g dry weight of 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.02% peptone, and 1.5% agar). Strains of *Cellumonas xylanilytica* and *Escherichia coli* JM109 were used as positive and negative controls, respectively, and were grown in LB medium overnight and spot plated on the same agar plates. All of the plates were incubated at 37 °C for 48 h and flooded with Gram's iodine solution for 3–5 min. The diameters of the halo region (D) and bacterial colony (d) were then measured on a centimeter scale. The hydrolysis activity was calculated as $(D/d)^2$, as described previously by Xiong et al. [32].

2.4. Determination of enzyme activities and reducing sugar

The samples were collected after 1, 2, 3, 4, 5, and 6 d of incubation and centrifuged at 12,000*g* for 3 min. The supernatants were used for the measurement of enzyme activities and reducing sugar content. Laccase activity was measured according to the method of Lu et al. [33], with some modifications. Briefly, 200 μl of reaction mixture containing 20 μl of diluted crude enzyme and 20 μl of 20 mM 2 2′-Azino-bis [3-ethylbenzothiazoline-6-sulfonate] (ABTS; Sigma, St. Louis, MO, USA) in a 0.1 M citrate buffer (pH 4.6) were incubated at 40 °C for 3 min. The absorbance was determined at 420 nm ($\varepsilon 420 = 36{,}000$ M^{-1} cm^{-1}) using a microplate spectrophotometer (Epoch, Bio Tek Instruments, Inc., Vermont, USA). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of substrate per minute.

The activities of CMCase, FPase, and xylanase were determined by detecting the released reducing sugar content from the

Table 1
The characteristics of the bacterial strains used in this study.

Isolates	Genus	Kinds of enzyme secretion	Accession no.	Source
A0	*Bacillus* sp.	CMCase, FPase, Xylanase	KP974676	Paudel and Qin [37]
A4	*Bacillus* sp.	CMCase, Xylanase, Laccase	KX665584	Guo et al. (unpublished)
AS2B	*Exiguobacterium* sp.	CMCase, Xylanase, Laccase	HM134063	Maki et al. [36]
CDS3	*Pseudomonas* sp.	CMCase	HE648161	Maki et al. [36]
CH2OS1	*Bacillus* sp.	CMCase, FPase, Xylanase	HQ331531	Maki et al. [36]
K1	*Bacillus* sp.	CMCase, FPase, Xylanase, Laccase	KP987117	Paudel and Qin [37]
X1	*Raoultella* sp.	CMCase, FPase, Xylanase	KY290273	In this study
X4	*Bacillus subtilis*	CMCase, FPase, Xylanase, Laccase	KY327801	In this study

substrates of carboxymethyl cellulose, filter paper, and beechwood xylan, respectively, using the 3,5-dinitrosalicylic acid (DNS) method, with some modifications [34]. Briefly, 30 μl of enzyme reaction mixture containing 10 μl of diluted crude enzyme and 20 μl of each substrate in 50 mM citrate buffer (pH 4.8) were incubated at 50 °C for 30 min. Next, 60 μl of DNS reagent were added to the above enzyme reaction mixture and boiled for 5 min. After cooling in a cold water bath, 210 μl of distilled water were mixed with the reaction mixture. The absorbance was then determined at 540 nm using a microplate spectrophotometer (Epoch, Bio Tek Instruments, Inc., Vermont, USA). Glucose was used as the standard to calculate the activities of CMCase and FPase, and xylose was used as the standard to calculate the activity of xylanase. One unit of activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar per minute. To measure the reducing sugar content, 30 μl of the enzyme reaction mixture were replaced with supernatant culture and mixed with 60 μl of DNS reagent. Glucose was used as the standard to calculate the content of reducing sugar.

2.5. Inoculum preparation

The fresh algae culture and supernatant of each strain were mixed in a ratio of 3:1 (v/v) and added to a 50 ml flask. Supernatant autoclaved at 121 °C for 15 min was used as a control. Incubation experiments were carried out at room temperature, with agitation at 200 rpm. After incubation for 0, 6, 12, 24, 36, and 48 h, the mixture was collected by centrifuging at 12,000*g* for 3 min, and the content of reducing sugar in the supernatant was measured using the DNS method, as described above (See the method section 2.4). At the end of the incubation, the cells were collected for lipid extraction.

In time-dependent experiments, only the supernatant from *Bacillus* sp. K1 was used for degrading algae due to its high lipid extraction efficiency and reducing sugar release. The inoculum experiment was prepared in the same manner as described above and stopped at different times (0, 6, 12, 24, 36, and 48 h). The algal cells were harvested for lipid extraction and microscope observations.

2.6. Lipid extraction

The total lipids from the wet algal biomass were extracted by chloroform:methanol (1:1, v/v) using the method of Bligh and Dyer [35], with some modifications. Briefly, 5.4 ml of the wet algal biomass (equal to 30 mg of dry biomass) were collected by centrifuging at 8000*g* for 10 min. The cell pellet was first extracted with a mixture of chloroform, methanol, and distilled water (1:2:0.8, v/v/v). The mixture was agitated by vortexing for 5 min and centrifuged at 8000*g* for 10 min. The supernatant was transferred to a pre-weighted Eppendorf tube (W_1 g), and the cell pellet was re-extracted with the mixture of chloroform and methanol (1:2, v/v). The mixture was centrifuged again, and the supernatant was collected in the same pre-weighted Eppendorf tube. Chloroform and water were then added to the supernatant to form a ratio of 1:1:0.9 (chloroform:methanol:distilled water, v/v/v). The mixture was mixed well and centrifuged at 8000*g* for 5 min. The top layer was then removed, and the bottom layer was evaporated and dried at 80 °C until reaching a constant weight (W_2 g). The total lipid content was calculated by subtracting W_1 from W_2 and was presented as a % of the dry weight.

2.7. Microscope and scanning electron microscope (SEM) observations

The microscope images of the algae cells before and after the treatment with the bacterial enzymes from *Bacillus* sp. K1 were observed using an Olympus BX51 microscope (Olympus Optical, Tokyo, Japan). The cells were collected after 6, 12, 24, 36, and 48 h of treatment and centrifuged at 4000*g* for 5 min. Untreated algae cells were used as a control. The cell pellets were washed three times with deionized water and then resuspended with an equal volume of deionized water. Then, 20 μl of appropriately diluted algae cells were plated on a clean glass slide. To ensure a representative count, each sample was divided into quarters, and two fields per each quarter were photographed with an Olympus BX51 microscope at 100. The cell numbers were determined visually through a compound light microscope. The relative intact cell number percentage (%) was calculated as the number of treated cells/number of untreated cells × 100.

For the SEM observations, appropriately diluted algae were directly dropped onto a clean silicon wafer and air dried for 20 min. The samples were then sputter-coated with gold and observed using the SEM (SEM, Hitachi AU-70, Tokyo, Japan).

2.8. Statistical analysis

Statistical analysis was carried out using a one-way analysis of variance with SPSS software (SPSS Inc., USA, Version 13.0). All the experiments were performed in triplicate, and the results are shown as mean ± SD. Differences between treatments were evaluated, and statistical significance was accepted at $P < 0.05$.

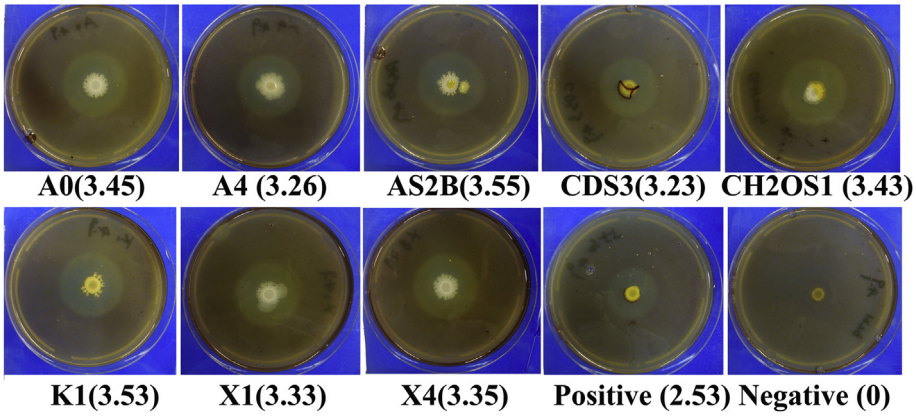

Fig. 1. Evaluation of enzymatic activity using algae biomass as carbon source by staining with Gram's iodine solution. Positive: *C. xylanilytica*; Negative: *E. coli*. The numbers in the brackets indicate the diameter of halos.

Table 2
Algal cell walls hydrolytic ability of eight different bacteria and two controls. Values represent mean ± SDs (n = 3). Differences letters indicate significant differences ($P < 0.05$) among different bacteria.

Isolates	Halo diameter (D, cm)	Colony diameter (d, cm)	Hydrolysis ability $(D/d)^2$
A0	3.45 ± 0.10a	0.91 ± 0.04b	14.4c
A4	3.26 ± 0.11b	0.98 ± 0.03a	11.1de
AS2B	3.55 ± 0.06a	0.89 ± 0.05b	15.9b
CDS3	3.23 ± 0.08b	0.94 ± 0.05ab	11.8d
CH2OS1	3.43 ± 0.13a	0.92 ± 0.06b	13.9c
K1	3.53 ± 0.14a	0.86 ± 0.04b	16.8a
X1	3.33 ± 0.19ab	0.99 ± 0.07a	11.3d
X4	3.35 ± 0.13ab	1.02 ± 0.04a	10.8e
C. xylanilytica	2.53 ± 0.05c	0.82 ± 0.02c	9.5f
E.coli BL21	–	0.65 ± 0.03d	–

3. Results and discussion

3.1. Hydrolysis of algal cell walls

The lignocellulolytic enzyme activity of eight different biomass-decomposing bacteria (five *Bacillus*, one *Exiguobacterium*, one *Pseudomonas*, and one *Raoultella* species) and two controls were evaluated on plates using algae as the sole carbon source. The strains A0, A4, AS2B, CDS3, CH20S1, and K1 were described in our previous research [31,36,37]. The strains X1 and X4 were isolated from forest soil and identified as *Raoultella* sp. and *Bacillus subtilis*, respectively (Fig. S1). Gram's iodine is considered as an easy, fast, and environmentally friendly qualitative method for screening microorganisms that produce extracellular cellulase on an agar plate [31]. Gram's iodine forms a bluish-black complex (halo region) with cellulose but not with hydrolyzed cellulose. In this study, all the strains produced a larger halo region than that produced by the industrial strain *C. xylanilytica*, indicating that the studied strains had excellent hydrolysis ability of algae biomass (Fig. 1). The hydrolysis activity of each bacterial colony was evaluated by calculating square of the halo diameter: colony diameter ratio to further confirm the algal degradation ability of these bacteria. The hydrolysis activity values of the strains ranged from 10.8 to 16.8. The hydrolysis activity value of the positive control strain *C. xylanilytica* was 9.5 (Table 2). Two bacterial strains were previously reported to have extremely high hydrolysis activity values and effectively degrade algal biomass to bioethanol and xylitol [32]. The mutants of *Bacillus amyloliquefaciens* for enhancing cellulase activity were screened according to the ratio between the hydrolysis halo diameter and the colony diameter [38].

3.2. Activities of lignocellulolytic enzymes secreted by the bacteria

All the studied strains showed superior CMCase activity, which differed markedly among the bacteria strains when wheat bran was used as the sole carbon source (Fig. 2A). Three *Bacillus* strains A0, A4, and CH20S1 and one *Raoultella* strain X1 secreted the most CMCase into the medium, with activities of 5.9, 5.5, 3.0, and 3.7 U ml^{-1}, respectively, after incubation for 2 d. The *Exiguobacterium* sp. AS2B and *Bacillus* sp. K1 exhibited the maximum CMCase activities, with values of 5.8 and 5.1 U ml^{-1}, respectively, after incubation for 3 d. *Pseudomonas* sp. CDS3 and *Bacillus subtilis* X4

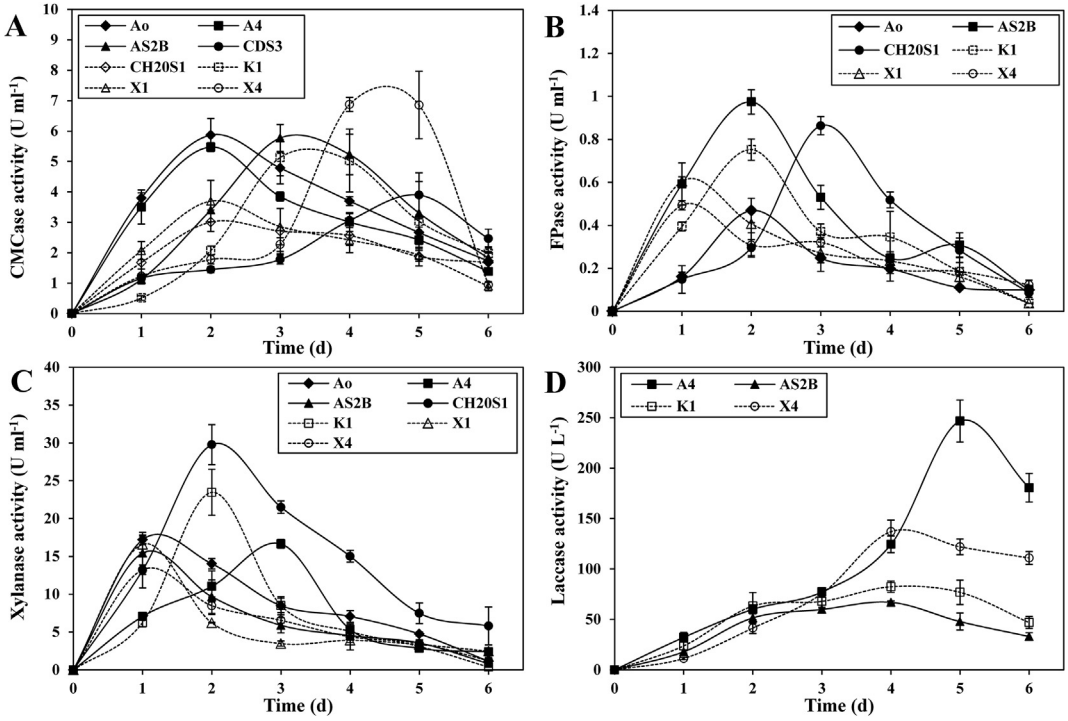

Fig. 2. Lignocellulolytic enzyme activities of different bacteria cultivated with wheat bran. (A) CMCase; (B) FPase; (C) Xylanase; and (D) Laccase activity.

Table 3
Yields of reducing sugars from fresh algae by different bacterial strains. Values represent mean ± SDs (n = 3). Differences letters indicate significant differences ($P < 0.05$) among different treatment time.

Treatment time (h)	The reducing sugars content from different strains (mg L^{-1})							
	A0	A4	AS2B	CDS3	CH2OS1	K1	X1	X4
0	36.6 ± 2.8e	31.7 ± 3.7e	40.6 ± 7.0e	32.5 ± 4.2d	52.7 ± 2.8d	43.0 ± 5.1e	70.5 ± 2.8f	40.6 ± 3.5e
6	90.5 ± 2.6d	91.3 ± 7.0d	105.1 ± 7.2d	105.1 ± 5.1c	135.0 ± 12.3c	148.1 ± 4.9d	136.6 ± 3.7e	119.6 ± 9.5d
12	126.1 ± 12.4c	103.4 ± 2.4c	111.5 ± 8.5d	127.7 ± 12.4b	143.9 ± 2.8c	160.8 ± 3.7c	149.6 ± 7.3d	145.5 ± 12.8c
24	141.7 ± 6.4c	129.4 ± 2.8b	135.0 ± 4.9c	135.8 ± 3.9b	163.3 ± 3.7b	173.1 ± 11.2bc	163.3 ± 5.1c	156.1 ± 3.7c
36	167.6 ± 3.7b	135.0 ± 2.4b	157.7 ± 1.4b	139.9 ± 4.2ab	172.3 ± 9.8b	186.6 ± 2.9b	175.5 ± 3.7b	166.6 ± 4.2b
48	186.0 ± 11.0a	148.8 ± 9.2a	204.6 ± 13.4a	145.5 ± 3.7a	203.0 ± 7.3a	209.0 ± 13.4a	197.3 ± 12.0a	185.2 ± 13.4a

showed the highest CMCase activities after incubation for 5 d, with values of 3.9 and 6.9 U ml^{-1}, respectively (Fig. 2A). FPase activities were detected in six strains, with activities in the A0, AS2B, CH20S1, K1, X1, and X4 strains of 0.47, 0.97, 0.86, 0.75, 0.60, and 0.49 6.9 U ml^{-1}, respectively, after 1–3 d of culture (Fig. 2B). When incubated using wheat bran as the sole carbon source, xylanase production was detected in the strains A0, A4, AS2B, CH20S1, K1, X1, and X4. Peak xylanase activity occurred on the first day in strains A0 (17.2 ml^{-1}), AS2B (15.4 ml^{-1}), and X4 (13.2 ml^{-1}). Peak activity occurred on the second day in strains CH20S1 (29.8 ml^{-1}), K1 (23.5 ml^{-1}), and X1 (16.6 ml^{-1}) and on the third day in strain A4 (11.0 ml^{-1}) (Fig. 2C). Four strains (A4, AS2B, K1, and X4) released laccase into the medium, with the maximum activity (246.7, 67.0, 82.4, and 137.0 U L^{-1}, respectively), detected on day 4 or 5 (Fig. 2D). Previous research reported that most *Bacillus* strains secreted CMCase and xylanase into medium and exhibited cellulose and xylan degradation abilities [39,40]. Some *Bacillus* strains were previously shown to produce laccase with high thermal and pH stability, which is widely used in lignin degradation, industrial wastewater treatment, soil bioremediation, and dye decolorization [41,42]. In addition, strains of *Exiguobacterium* and *Pseudomonas* were reported to produce extracellular lignocellulolytic enzymes, which can be used to decompose various types of biomass [43,44].

3.3. Reducing sugar production

The reducing sugar content significantly increased ($P < 0.05$) after incubation with fresh algae and in the bacterial enzyme culture with time (Table 3). After 6 h of incubation, the reducing sugar content increased 2–3-fold as compared to that of the controls. With all the strains, the highest reducing sugar content was obtained at the end of the treatment, with the maximum content obtained using strains AS2B, CH2OS1, and K1, which produced 204.6, 203.0, and 209.0 mg L^{-1} reducing sugar, respectively (Table 3). Algae are typically constituted of polysaccharides (cellulose, uronic acid, protein, mannose, and xylan), trilaminar layers of algaenan, and glycoproteins [45]. Unlike red algae and brown algae, green algae have a high cellulose content in their cell walls [46]. Some bacterial enzymes, such as exoglucanases, endoglucanases, and xylanase, can efficiency hydrolyze cellulose and hemicellulose to form reducing sugars [25]. In the present study, the strains AS2B, CH2OS1, and K1 showed excellent CMCase, FPase, and xylanase production, thus forming more reducing sugars than the other strains (Fig. 2a–c and Table 3). This was similar to that of an enzyme mixture (α-amylase, cellulose, and β-glucosidase), which produced 10% more reducing sugar from the green algae *Enteromorpha* genus than α-amylase alone [46].

3.4. Lipid extraction

The algal biomass samples were collected after co-culturing with the bacterial medium to evaluate the lipid extraction efficiency of the different biomass-degrading bacteria. As expected, due to cell wall degradation by the bacterial enzymes, the lipid extraction efficiency of all the bacteria was markedly improved ($P < 0.05$) after 48 h of the treatment compared to that of the controls (Fig. 3). This result indicated that cell wall degradation aided the extraction of intracellular lipids from the algae. However, the efficiency of lipid extraction differed significantly among the studied strains. The highest lipid content was obtained from strain K1, with an increment ratio of 43.9%, and the lowest lipid content was found with strain CDS3, with a value of 10.4% (Fig. 3). The lipid content increased most with strains A0, A4, AS2B, CH20S1, X1, and

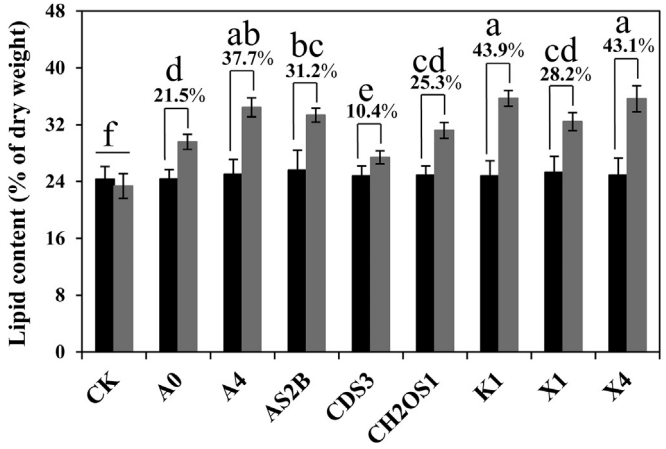

Fig. 3. Effects of bacterial strains on algal lipid extraction from wet biomass of algae after 48 h of incubation. Values represent mean ± SDs (n = 3). Different letters indicate significant differences ($P < 0.05$) among different bacteria.

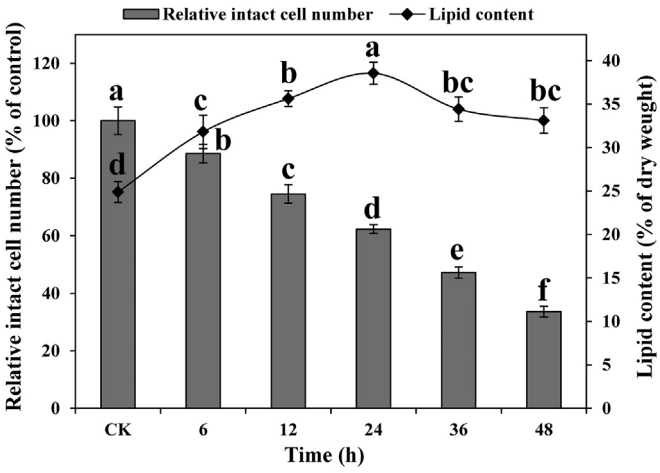

Fig. 4. Effects of *Bacillus* sp. K1 on algae cell wall degradation and lipid extraction from wet biomass of algae after 0, 6, 12, 24, 36 and 48 h of treatment. Values represent mean ± SDs (n = 3). Differences letters indicate significant differences ($P < 0.05$) among different treatment time.

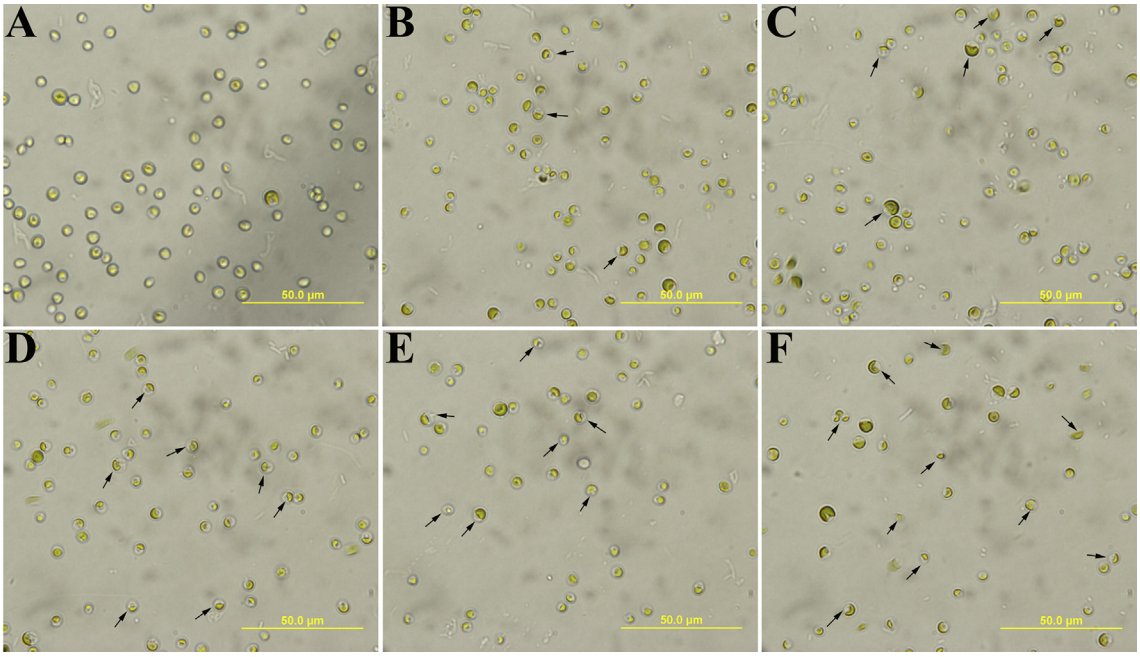

Fig. 5. Microscope images of algae cells before and after treatment using *Bacillus* sp. K1. The arrows indicate the broken cells after treatment.

X4, with rises of 21.5, 37.7, 31.2, 25.3, 28.2, and 43.1%, respectively. Cho et al. [23] used a mixture of cellulases and β-glucosidases to hydrolyze *Chlorella vulgaris* for 72 h. They found that the lipid extraction yield was improved by 29–73%, depending on the solvents used, as compared to when no enzymatic hydrolysis process was involved. Lipid concentrations using enzymatic lysis of *Chlorella vulgaris* cells by snailase, lysozyme, and cellulose were 1.4, 4.5, and 4.8 times higher than that of untreated cells, respectively [27]. In the present study, the lipid extraction efficiency of the laccase-producing strains (A4, AS2B, K1, and X4) was higher than that of the nonlaccase-producing strains (Fig. 3). The lipid extraction efficiency of these strains may be related to the decomposition of chlorophyll, mediated by laccase. The following may explain the higher lipid content. First, although there is almost no lignin in algal biomass, the thin trilaminar outer wall of all freshwater microalgae produces a highly aliphatic algaenan [13,47]. This wall may be degraded by laccase, thereby enabling enzyme attack by other enzymes [13,47]. Second, the decomposition of chlorophyll can disrupt the competitive reaction with lipids to dissolve into organic solvent [22,48]. As a result, nonpolar solvents can more easily gain access to lipids. Third, the secondary products from chlorophyll degradation may be extracted as lipids because chlorophyll contains a phytol chain [49].

3.5. Effect of the pretreatment on disruption of the algal cell walls

To evaluate the effects of the incubation time on the disruption of the algal cell membrane and total lipid extraction yield, a supernatant of the *Bacillus* sp. K1 strain, which resulted in a higher reducing sugar content and better lipid extraction efficiency, was selected to co-culture with fresh algae for 6, 12, 24, 36, and 48 h. The results showed that the lipid extraction yield significantly increased at all the measured time points and reached a peak after 24 h, with an increment of 52.3% compared to that of the control (Fig. 4). Disruption of the algal cell membrane was visualized and analyzed by counting 10 random views under an Olympus BX51 microscope. In the nontreatment, the images indicated that the cells were intact and mostly spherical in shape. In contrast, in the pretreatment, the images showed a large amount of cell debris and many small and transparent moving particles (Fig. 5). These results were very similar to those reported earlier when microalgae were treated with 3% sulfuric acid at 160 °C for 15 min [50]. The SEM images showed that the algal cells were paralyzed and fragile after the pretreatment (Fig. S2), indicating that lipid release was much easier than in the intact cells. The number of intact cells decreased markedly in accordance with an increment in the incubation time. The number of intact cells decreased 88.6, 74.5, 62.3, 47.3, and 33.7% after 6, 12, 24, 36 and 48 h of incubation, respectively, compared to that of the untreated controls (Figs. 4 and 5). The sharp reduction in cell numbers and release of lipids into the liquor may explain the decrease in the lipid extraction yield after 36 and 48 h. The decline in the number of intact cells was in accordance with an increase in the reducing sugar content (Fig. 4 and Table 3), suggesting that co-cultivation of a supernatant of the *Bacillus* K1 strain with algal cells can efficiently disrupt and decompose algal cell walls. A previous study demonstrated that the collapse in the cell wall structure caused by enzymatic hydrolysis made it easier to recover lipids [23]. Another study found that free nitrous acid pretreatment resulted in algal cell wall and membrane disruption, thus enhancing the lipid extraction yield from algae [22]. The algal cell walls are typically constituted of a tri-layered structure, which are three times stronger than that of plant cell walls [14,15]. Therefore, disruption of the algae cell walls is necessary to release more lipids during the lipid extraction process [17].

4. Conclusions

This study showed that algal cell walls can be disrupted by lignocellulolytic enzymes secreted by bacteria. The total lipid extraction yield from algae was enhanced by 10.4–43.9%, depending on the bacteria used. Laccase-producing bacteria (A4, AS2B, K1, and X4) seemed to aid lipid release from algal cells, although some of the bacteria did not produce more reducing sugars. In a time-course experiment with *Bacillus* sp. K1, which was selected due to its high reducing sugar production ability and lipid extraction efficiency, the maximum lipid content was obtained after 24 h of incubation, with about a 40% cell disruption rate.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.renene.2017.03.025.

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