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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 exoglucanases (CMCase), endoglucanases (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 exoglucanases (CMCase), endoglucanases (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