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Research Article

Active extracellular substances of *Bacillus thuringiensis* ITRI-G1 induce microalgae self-disruption for microalgal biofuel

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Microalgal cultures are a clean and sustainable means to use solar energy for CO₂ fixation and fuel production. Microalgae grow efficiently and are rich in oil, but recovering that oil is typically expensive and consumes much energy. Therefore, effective and low-cost techniques for microalgal disruption and oil or lipid extraction are required by the algal biofuel industry. This study introduces a novel technique that uses active extracellular substances to induce microalgal cell disruption. A bacterium indigenous to Taiwan, *Bacillus thuringiensis*, was used to produce the active extracellular substances, which were volatile compounds with high thermal stability. Approximately 74% of fresh microalgal cells were disrupted after a 12-h treatment with the active extracellular substances. Algal lipid extraction efficiency was improved and the oil extraction time was decreased by approximately 37.5% compared with the control treatment. The substances effectively disrupted fresh microalgal cells but not dehydrated microalgal cells. An analysis of microalgal DNA from fresh cells after disruption treatment demonstrated typical DNA laddering, indicating that disruption may have resulted from programmed cell death. This study revealed that biological treatments are environmentally friendly methods for increasing microalgal lipid extraction efficiency, and introduced a microalgal cell self-disruption mechanism.

Keywords: *Bacillus thuringiensis* / Cell disruption / Lipid extraction / Microalgae / Programmed cell death

Received: August 17, 2016; revised: November 8, 2016; accepted: November 21, 2016

DOI: 10.1002/elsc.201600194

1 Introduction

Development of biofuels has been an area of active research for decades because of a desire to reduce dependence on fossil fuels. Microalgal biofuel is a promising third-generation biofuel. However, because the current procedures for extracting oil from microalgal biomass are expensive, new methods are required to achieve its commercialization [1, 2]. The mechanical disruption of microalgal cells is a critical step in the process because it consumes large amounts of energy [1]. The disruption of microalgal cell walls before lipid extraction is generally necessary to release the internal lipids.

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Abbreviation: ITRI, Industrial Technology Research Institute; PCD, programmed cell death

Enzymatic methods have recently been developed as viable alternatives to mechanical or chemical cell disruption methods, because they have several advantages including reduced energy requirements, high enzymatic reaction selectivity, mild temperature, and pH requirements, and do not involve inhibiting by-products [3–5]. Therefore, enzymatic methods are less energy intensive and more environmentally sustainable than conventional mechanical, thermal, or chemical methods. Nevertheless, enzymatic treatments for disrupting microalgal biomass are costly. Moreover, formulating enzyme cocktails with optimal compositions and dosages is difficult. To reduce the cost of extraction, induced autolysis of microalgae has recently been proposed as a promising cell disruption method [6]. Microalgal cell disruption using algicidal microorganisms is a feasible, accessible, and cost-effective biological alternative for biofuel production. In this study, the identification, characterization, and algicidal effect of the algicidal bacterium *Bacillus thuringiensis* Industrial Technology Research Institute (ITRI)-G1 on microalgal cell disruption is reported.

2 Materials and methods

2.1 Algal cultures

A culture of *Chlorella* sp. was maintained in BG11 medium (ATCC medium 616) and aerated at 0.1 vvm (1–2% CO₂) in glass bottles at 25°C and pH 7, with an illumination cycle of 12 h of darkness followed by 12 h under a cool white fluorescent light (3000 lx). CO₂ gas at a rate of 1 vvm was used for aeration. The concentration 10 g/L (dry algae cell/liquid of active substances) was used in the algicidal action.

2.2 Identification of bacteria by phylogenetic analysis

Morphological characteristics were determined by Gram staining. A bacterial strain was identified using the sequence homology of 16S rDNA; 16S rDNA of the strain was amplified using polymerase chain reaction, and the sequence was compared with that of the 16S rDNA of other known bacteria. The Vitek identification system was used and *gyrB* gene sequences were identified to confirm the identity of the bacterium as *B. thuringiensis*. The presence of *cry* gene fragments (*cryIAa*, *cryIAb*, *cryIAc*) was confirmed using gel electrophoresis. *Bacillus thuringiensis* ITRI-G1 was deposited in the Leibniz-Institut DSMZ GmbH (Germany) as DSM 29807.

2.3 Cultivation of *B. thuringiensis* ITRI-G1 and purification of active extracellular substances

ITRI-G1 colonies were inoculated in 500 mL flasks containing 250 mL neurobasal medium (5 g/L peptone and 3 g/L yeast extract) and maintained at 30°C on a gyratory shaker at 150 rpm. The broth was harvested after 3 days of cultivation and centrifuged to obtain the supernatant. A rotary evaporator was used at 50°C to collect the liquid in the reservoir for 2 h at 70 mbar.

2.4 Algicidal effects of bacterium ITRI-G1

Determination of the algicidal mechanism was performed in a 1.5 mL Eppendorf tube. One milliliter of a solution of active extracellular substances was introduced into the Eppendorf tube, to which 0.01 g of algal culture was added and mixed thoroughly. The algicidal activity of the ITRI-G1 strain was observed over a 24-h period after recording the initial activity. The OD of the supernatants of the solution (centrifuged at 10 000 × *g* for 5 min) was measured at 280 nm to monitor the change in protein concentration. Independent experiments were performed at least twice.

2.5 Microalgal oil extraction

A 0.2-g sample of microalgae was added to 10 mL of the active extracellular substance solution and allowed to react for 1 day. The microalgae were then collected for Soxhlet extraction of oil. Independent experiments were carried out at least twice.

Figure 1. Cell disruption activity of ITRI-G1 under different evaporation conditions.

2.6 DNA laddering

The genomic DNA of *Chlorella vulgaris* was extracted according to a previously described method [7]. In total, 3×10^7 cells per sample were spun down after 2 min of centrifugation at 3700 × *g*. Cell pellets were collected and ground to a fine powder in liquid nitrogen. The disrupted cells were then treated with DNA extraction buffer (2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0, and 10 mM β-mercaptoethanol) at 65°C for 20 min. The extracted DNA was then purified through two rounds of phenol and chloroform:isoamyl alcohol (24:1 v/v) extraction to remove cell debris and proteins, and then precipitated with ice-cold isopropanol. The precipitated DNA was washed twice using 70% v/v ethanol. The air-dried DNA pellet was dissolved in 25 μL TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA). The DNA samples were then treated with DNase-free RNase A to remove RNA contamination. Finally, 3 μg of each DNA sample was resolved on 1.8% agarose gel and stained with ethidium bromide.

3 Results and discussion

3.1 Microalgal cell disruption by volatile active extracellular substances from *B. thuringiensis*

A microalgal cell lysing bacterium was isolated from a pond, which was identified to be *B. thuringiensis* ITRI-G1 by the Biore-source Collection Research Center (Food Industry Research and Development Institute, Taiwan). The substances produced by the ITRI-G1 were isolated using a rotary evaporator in the reservoir. A solution containing these volatile substances disrupts *C. vulgaris* cells. The broth remaining in the pot, however, cannot disrupt the microalgal cells. It is possible that some volatile active extracellular substances secreted by ITRI-G1 are lethal to microalgae. Monitoring the OD value of the treated microalgal solution at 280 nm demonstrated that the protein concentration increased gradually over time. The microalgal cell membrane was damaged and the intracellular cytosol and chlorophyll flowed away (Fig. 1). The cell wall was completely disrupted after 8 h (Fig. 2).

Figure 2. (A) Before and (B) after microalgae reacted with active extracellular substances.

Table 1. Variation in the ratio of available and membrane-damaged cells that are counted using a microscope

Disruption treatment time, h	0 (%)	8 (%)	12 (%)	24 (%)
The ratio of microalgae cell with intact shape	100	98	97	92
The ratio of microalgae cell with intact shape and chlorophyll	93	54	26	15
Disrupted microalgae cell	7	46	74	85

Table 1 presents the change to the ratio of available cells to membrane-damaged cells with time. After 12 h of disruption treatment, only 26% of the cells remained intact in terms of their shape and chlorophyll, which represents the percentage of available cell. Approximately 74% of the cells showed evidence of cell death and membrane damage, causing chlorophyll and other intracellular material to flow out. However, 97% of the cells retained their shape. Therefore, approximately 71% of the microalgal cells showed evidence of cell death but retained their shape.

The cell disruption caused by the volatile substances of ITRI-G1 was not similar to that caused by enzymatic lysing. During enzymatic lysing, hydrolase attacks and disrupts the microalgal cell wall, and most of the disrupted cells break. By contrast, the volatile substances of ITRI-G1 caused death in most cells but engendered fewer cell disruptions.

3.2 Microalgal cell disruption improved oil extraction efficiency

Treating microalgal cells with active extracellular substances from *B. thuringiensis* ITRI-G1 caused cell death. However, microalgal cell wall disruption is less easily observed than cell death. Whether the active extracellular substances improved extraction efficiency required further confirmation.

The Soxhlet extraction process was used to examine whether cell disruption improved oil extraction efficiency. Fig. 3 illustrates that the treatment of microalgal cells with active extracellular substances from *B. thuringiensis* ITRI-G1 clearly improved the oil extraction efficiency when compared with the control, in which the algal cell were not disrupted. Extraction of 80% of the total oil through the Soxhlet process required approximately 8 and 5 h in untreated and treated microalgal cells, respectively. Cell disruption using the active extracellular substances reduced the oil extraction time by approximately 37.5%.

Oil extraction from the cells disrupted using the active extracellular substances required approximately 25% more time

Figure 3. Oil extraction rate of microalgae treated with active substances (AS) and bead mill process (bead).

than that from cells disrupted using a bead mill. This may be because the cells retained their shapes after the disruption induced by the active extracellular substances; therefore, the extraction was hindered despite the cells' treatment. The bead mill process, however, destroyed the entire microalgal cell and oil extraction was not hindered. Thus, mechanical disruption could result in improved oil extraction.

Mechanical disruption consumes a large amount of energy. Lee et al. [8] determined the energy consumption of various mechanical disruption processes. The energy consumption of the bead mill process was approximately 120 000 kcal/kg of algae, whereas the energy consumption of cell disruption using active extracellular substances from *B. thuringiensis* ITRI-G1 was relatively low at approximately 506 kcal/kg of algae. Of this amount, almost all was consumed during the production of the active extracellular substances, not by the actual cell disruption itself. Cell disruption using active extracellular substances can thus reduce energy consumption and processing time during microalgal

Figure 4. Disruption level of fresh and dehydrated cells of *Chlorella vulgaris* CNW-11 treated with active substances indicated by OD₂₈₀.

Figure 5. Effects of stirring on algae cell disruption induced by active extracellular substances.

biofuel production, which are two of the major barriers to the commercialization of microalgal biofuels.

3.3 Active extracellular substances do not cause disruption in dehydrated microalgal cells

To confirm that the cell disruption was not caused by cell wall destruction, dehydrated, intact microalgal cells that retained their shape and contained cytoplasm were prepared by freeze drying. This study hypothesized that the dehydrated cells would not be attacked by the active extracellular substances, the entire cell would not be broken, and the protein would not be detected.

The results illustrated in Fig. 4 support this hypothesis. Disruption treatment on fresh and dehydrated microalgal cells demonstrated that only the fresh cells released proteins that were detected by measuring the OD at 280 nm. The OD at 280 nm increased from 0.2 to 5.0 over 24 h of disruption treatment using active extracellular substances. By contrast, the dehydrated

microalgal cells treated with active substances did not show an increase in protein concentration, with the OD at 280 nm increasing from 0.2 to 0.4 over 24 h of disruption treatment, which was similar to the control process in which the OD at 280 nm increased from 0.2 to 0.3 over 24 h.

The active extracellular substances did not attack the microalgal cell wall directly. Therefore, only fresh microalgal cells could react with the active extracellular substances and induce them to undergo disruption.

3.4 Stirring does not affect cell disruption

Traditional algae cell wall disruption using hydrolysis enzymes requires stirring to improve mass transfer and increase hydrolysis reaction efficiency. However, this was not required during disruption induced by active extracellular substances. As Fig. 5 shows, Low speed stirring at 50 rpm was performed, cell disruption increased by only 17.4% compared with cell disruption when no stirring was performed. Increasing stirring speed beyond this decreased cell disruption. The OD₂₈₀ value of the protein released by the disrupted algae cells decreased from 3.87 to 1.66 when stirring was performed at 200 rpm.

During enzymatic hydrolysis disruption, stirring was required for improving the contact between the enzymes and algae cell walls. However, disruption induced using active extracellular substances was not reliant on the same mechanism. Most chemical and biochemical reactions were induced in the algal cells, and were not affected by the stirring outside. Only a little mass transfer was required to improve the contact between the active extracellular substances and algae cells at the initial disruption stage to induce self-disruption.

Too intense stirring decreased the induced self-disruption. Some algae cells uptake cytoplasm as food that is released from disrupted algae cells. Therefore, the algae cells produce energy to resist the self-disruption reaction.

This phenomenon is advantageous, because it decreases the energy consumed during algae cell disruption. Disruption induced by active extracellular substances can be left to proceed without stirring. This is why the energy consumption of cell disruption using active extracellular substances was relatively low at only 506 kcal/kg of algae, almost all of which is consumed during the production of the active extracellular substances.

3.5 Active extracellular substances from ITRI-G1 induce DNA fragmentation in *C. vulgaris*

Because the active extracellular substances did not affect the dehydrated cells, it is likely that they induced cell disruption through programmed cell death (PCD). DNA laddering due to programmed DNA degradation into fragments of approximately 180 bp is a hallmark of numerous types of PCD, including apoptosis. DNA laddering was observed after 2 h of treatment with the active extracellular substances, became pronounced between the 12th and 16th hours of treatment (Fig. 6). After 24 h of treatment, major DNA degradation was observed. A minor DNA-laddering pattern was also observed in the control treatment (treatment with distilled water), which may be attributed

Figure 6. DNA laddering in active extracellular substance treated *Chlorella vulgaris*. DNA was isolated from a 50% active extracellular substance treated suspension of *C. vulgaris* cells. Analysis was performed after 2, 4, 8, 12, 16, and 24 h of treatment. DNA was subjected to agarose gel electrophoresis using 3 μ g DNA per lane. The arrow heads indicate the DNA-laddering pattern. Lane 1: M, size marker; lane 2: C, control treatment with distilled water for 24 h; lanes 3–8: time points after application of active extracellular substances.

to cell death caused by the hypotonic conditions. However, the major DNA-laddering pattern induced by the active extracellular substances indicates that they induced microalgal PCD. During PCD, many hydrolytic enzymes are induced to degrade cellular components, including cell walls. These findings may explain why active extracellular substances do not degrade dehydrated cells, and may signal a new direction for finding efficient cell disruption methods before oil extraction.

4 Concluding remarks

This study introduced a novel microalgal cell disruption process using the active extracellular substances produced by *B. thuringiensis* ITRI-G1. These substances induced the self-disruption of microalgae cells, but did not attack the cell walls directly, as is the case when hydrolyzing enzymes are used. Approximately 74% of microalgal cells were disrupted after a 12-h treatment. Cell disruption using active extracellular substances reduced the oil extraction time by 37.5%. Because the process employed cell self-disruption, the energy consumption of microalgal cell disruption was only 506 kcal/kg of algae, which is low enough to allow for microalgae biofuel commercialization. Through the identification of DNA laddering in the algae cells,

the active extracellular substances were demonstrated to induce PCD.

Practical application

This study used *Bacillus thuringiensis* ITRI-G1 to produce active extracellular substances for microalgae cell disruption. Approximately 74% of microalgal cells were disrupted after a 12-h treatment. Cell disruption using the active extracellular substances reduced oil extraction time by approximately 37.5%. Because of this induced disruption time, the energy consumption of the microalgae cell disruption was decreased, which is advantageous for microalgae biofuel commercialization. This study discovered that the induced microalgae cell self-disruption was a programmed cell death mechanism, as evidenced by DNA laddering of algae cells.

This work was supported by the Bureau of Energy, Ministry of Economic Affairs, R.O.C.

The authors have declared no conflicts of interest.

5 References

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