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Kinetics Study for the Recombinant Cellulosome to the Degradation of Chlorella Cell Residuals

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***Abstract*—**In this study, lipid-deprived residuals of microalgae were hydrolyzed for the production of reducing sugars by using the recombinant *Bacillus* cellulosome, carrying eight genes from the *Clostridium thermocellum* ATCC27405. The obtained cellulosome was found to exist mostly in the broth supernatant with a cellulosome activity of 2.4 U/mL. Furthermore, the Michaelis-Menten constant (K_m) and V_{max} of cellulosome were found to be 14.832 g/L and 3.522 U/mL. The activation energy of the cellulosome to hydrolyze microalgae LDRs was calculated as 32.804 kJ/mol.

***Keywords*—**Lipid-deprived residuals of microalgae, cellulosome, cellulose, reducing sugars, kinetics.

I. Introduction

RECENTLY marine microalgae have received a great attention owing to their potentials in the application of biofuel and bioenergy productions. Microalgae can be applied as food additives, alternative carbohydrate sources and mitigation of carbon dioxide via the photoautotrophic reaction [1]-[5]. Many reports have mentioned about the use of microalgae oils as the raw materials for biodiesel production [6]-[9]. Some of the microalgae were screened as the oil-rich materials for oils suppliers. Sometimes, the physical or chemical pretreatments to disrupt algae cell walls were a must before oils extraction [10]. After the extraction, a large amount of lipid-deprived residues (LDRs) would be produced as the wastes, which might still contain a lot of carbohydrate substances suitable to be used as the substrates in the production of biofuels such as hydrogen, bioethanol, and methane [11]-[14]. The LDRs are considered to be the high potential substances in the biofuel development [15].

The use of the microbial cellulases is considered to be an environment-friendly approach to the hydrolysis of cellulosic materials [16] due to the advantages such as milder pH and temperature conditions, high selectivity, and fewer side-products [3], [17]. The Chlorella cell walls contain a large amount of cellulose, which exists as crystalline and amorphous forms. With its insolubility and heterogeneity, the native cellulose is highly resistant to enzymatic hydrolysis[18]. The multi-enzyme system of cellulosome from microorganisms might provide a solution to circumvent this issue. In the literature, Ho et al. have constructed an artificial cellulosome carrying eight *Clostridium thermocellum* cellulosomal genes, including one scaffolding protein gene (*cipA*), one cell-surface anchor gene (*sdbA*), two exo-glucosidase genes (*celK* and *celS*), two endoglucanase genes (*celA* and *celR*), and two xylanase genes (*xynC* and *xynZ*) into *Bacillus subtilis* [19]. A process for cellulosic bioethanol production was also conducted via the *Bacillus/yeast* co-culture system. The simultaneous saccharification and fermentation process by using recombinant *Bacillus subtilis* with the multi-enzyme complex cellulosome was illustrated [19]. However, the detail properties of the cellulosome enzyme reaction were still unveiled. Meanwhile, many studies mentioned about the degradation of Chlorella cells with cellulases or immobilized cellulases. None of them proposed the degradation reaction using recombinant microbial cellulosome.

In this study, the LDRs obtained from an indigenous strain of *Chlorella vulgaris* ESP-31 were used as the substrates for carbohydrate degradation reaction [15]. A recombinant *B. subtilis* W800N strain with the cell membrane anchoring cellulosome was applied in this reaction. The Chlorella LDRs degradation by using the recombinant cellulosome was the first time revealed. The hydrolysis efficiency of the cellulosome was tested. The kinetics mechanism of the cellulosomal enzymes was studied. The compositions of the reducing sugars produced were also analyzed and discussed.

II. Materials and Methods

A. Preparation of Substrate (Algal Residue)

The lipid-deprived residuals (LDRs) from microalgae *Chlorella vulgaris* ESP-31 were a gift from Prof. Jo-Shu Chang in National Cheng Kung University, Taiwan. The dry LDRs were washed twice with distilled water and centrifuged at the gravity force of 3000 × g for 15 min, followed by lyophilization for 24 h. To prepare various substrate concentrations, the dry LDRs were weighted and resuspended in 1L sodium acetate buffer (50mM, pH5.0) as a substrate for enzymatic reaction.

B. Strain Cultivation

Bacillus subtilis WB800N, harboring an artificial cellulosome gene based on the proteome-wide order of *Clostridium thermocellum* ATCC27405, was a gift from Prof. Chieh-Chen Huang in National Chung Hsing University, Taiwan [15]. The recombinant *B. subtlis* was cultivated in 100 mL of Luria-Bertani (LB) medium composed of 5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl, plus 1 □g/mL tetracycline and grown at 37℃ and 200 rpm for 16 h. 10% of the harvested broth was used to inoculate 100 mL of LB medium culture as the main culture. This cultivation was carried out at 37℃ and 200 rpm. When the culture cells reached an optical density (OD_{600}) value of 0.6, isopropyl

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β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM, and the culture was further incubated at 37℃ for 12 h.

C. Cell Fractionation

After induction, the harvested broth (100 mL) was centrifuged at 3000×g for 10 min to obtain the broth supernatant and the whole cells pellet, respectively. Afterwards, the pellet was resuspended in sodium acetate buffer (50 mM, pH 6) to reach a cells solution with an OD_{600} of 6. The cell fractionation was performed according to the modified method from [20]. Cells solution (10 mL, OD_{600} =6) was disrupted via the French press (NN11 4SD, Constant Systems Limited, UK). The disrupted cell solution was centrifuged at 3000 ×g for 30 min at 4℃, and the soluble proteins of cytoplasmic fraction and cell debris were collected and used in the enzymatic reaction.

D. Cellulosome Activity Assay

Various fractions of cellulosome obtained in Section C were separately used to hydrolyze microalgae LDRs (100 g/L) at the conditions of 200 rpm and 60℃ for 10 min. The sample was taken for DNS reducing sugar analysis [20]. Glucose concentrations from 0.4 to 1.5 mg/mL were used as the standard to build the calibration curve. One unit of cellulosome activity was defined as 1 μmol glucose-equivalent per minute.

E. Kinetics Parameters Determination

To calculate the maximum reaction rate (V_{max}) and the Michaelis-Menten constant (K_m), the Lineweaver-Burk plot with $1/V$ versus $1/S$ was drawn, where V is the reaction rate in (U/mL) and S is the substrate concentration (g/L) [20].

As to the activation energy, the Arrhenius equation (1) was used [21].

$$V = k \times e^{-\frac{Ea}{RT}} \quad (1)$$

where V is the reaction rate of cellulosome in (U/min), E_a is the activation energy (kJ/mol) and R is the universal gas constant (8.314 J/mol K). The plot of ln(V) versus $1/T$ gave a linear line with the slope of - E_a /R.

III. Results and Discussion

A. Hydrolysis of microalgae LDRs via Cellulosome

To practice the hydrolysis of microalgae LDRs with the cellulosome, the *B. subtilis* WB800N carrying recombinant cellulosome from *C. thermocellum* ATCC27405 was applied with the substrate from microalgae LDRs (*C. vulgaris* ESP-31LDRs). Northcote et al. have analyzed the cell wall composition of *Chlorella pyrenoidosa* and shown that it contained 15.4% α-cellulose and 31.0% hemicellulose [22]. Fu et al. using immobilized cellulase for the hydrolysis of cell wall of *Chlorella* sp. to obtain a yield of 58% of hydrolyzed glucose relative to total sugar content [3]. Chen et al. analyzed carbohydrate components of *C. vulgaris* ESP-31 and found that glucose (mainly from cellulose and starch), xylose, galactose, arabinose and rhamnose are the major components [15]. A recombinant cellulosomal *Bacillus subtilis* carrying various cellulases were achieved to obtain 4 g/L reducing sugars from hydrolyze Napier grass [19]. The LDRs obtained from microalgae *C. vulgaris* ESP-31LDRs was analyzed with HPLC and found to contain glucose (58 %), xylose and arabinose (less than) (data not shown). All of these sugars are reducing sugars. Therefore, the reducing sugars produced in the reaction were detected and used to define the activity of the cellulosome.

In order to check the localization of cellulosome in the culture, the harvested broth was centrifugated into whole cells and supernatant, and the whole cells were further disrupted via the disrupted cells and centrifugated into cytoplasmic and cell debris parts. All of the four parts were used separately to hydrolyze microalgae LDRs (100 g/L) at conditions of 200 rpm and 60℃ [19]. The results are shown in Fig. 1. A cellulosome activity of 0.15 U was found to exist in the concentrated whole cells fraction (OD_{600}=6 or 23.8 mg CDW/mL). Furthermore, as compared the cytoplasm activity (0.001 U/mL) to the cell debris activity (0.056 U/mL), the major location of cellulosome was found to settle in the cell walls (Fig. 1). This proved that the artificial cellulosome carrying a cell-surface anchoring gene (sdbA) might help to locate celluloseom in the whole cells. However, it is interesting to find that the highest cellulosome activity of 2.4 U/mL was obtained in the broth supernatant (culture harvested at OD_{600}=0.8 or 4.8 mg CDW/mL). This meant that the *B. subtilis* could carry only few of the cellulosome in its membrane as compared to that being secreted out into the supernatant. In other words, the anchoring gene motif did function to harbor cellulosome in the membrane of *B. subtilis*; however, the poor activity demonstrates the low anchoring efficiency. The large-molecular-size of cellulosome and the less surface area of the membrane are likely the reasons for the low activity expressed in the whole cells.

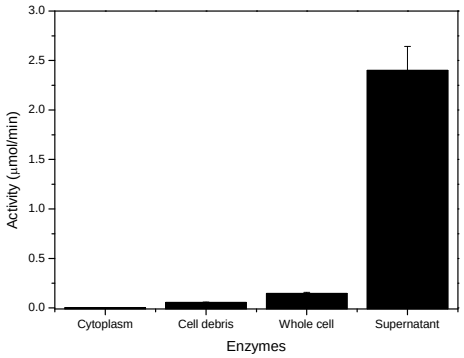

Fig. 1 Hydrolysis of microalgae LDRs via Bacillus cultivation fractionation

B. pH and Temperature Effect on LDRs Hydrolysis

Effect of enzymatic reaction pH value and temperature on the hydrolysis performance was examined. The results indicate that the cellulosome had a high and stable activity performance within the pH values between 6 and 9 as shown in Fig. 2. This result proves the obtained cellulosome is much tolerant to the alkali condition.

Effect of enzymatic reaction temperature on the hydrolysis

performance was investigated. According the experimental results in Fig. 3, the activity was significantly increased from 25 to 60℃, and then kept level-off at temperature higher than 60℃. This demonstrates the cultivated cellulosome is the thermophilic enzyme following the original gene codes expression of *C. thermocellum* ATCC27405.

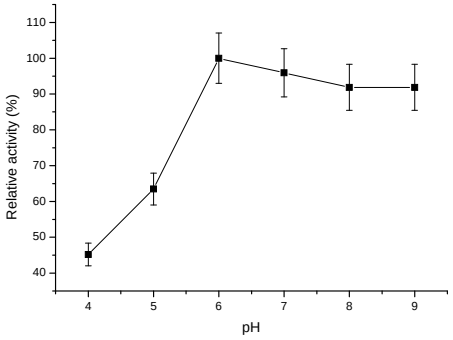

Fig. 2 Effect of pH value for hydrolytic waste microalgae LPRs

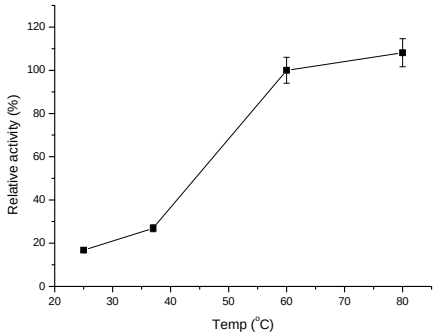

Fig. 3 Effect of temperature for hydrolytic waste microalgae LPRS

C. Kinetics Study on the cellulosomes

To calculate the activation energy, cellulosome reactions under temperatures range from 25 to 80℃ were performed. The activation energy value for the cellulosome was calculated to be 32.804 kJ/ mol (Table I).

TABLE I
KINETICS PARAMETERS OF CELLULOSOMES ACTIVITY

Location of cellulosome	Km (g/L)	Vmax (U/mL)	Ea (kJ/mol)
Whole cell	34.6	0.18	-
Supernatant	14.8	3.52	32.8

To study the enzymatic performance, the kinetics parameters of the Michaelise – Menten equation were separately determined for the cellulosome existing at the whole cells and the broth supernatant. Experiments were carried out using microalgae LDRs concentrations from 10 to 100 g/L and operated at 60℃. The K_m and V_{max} values were calculated from Lineweavere – Burk plots and the results are shown in Table I. The V_{max} values for the whole cells and supernatant were 0.18 and 3.52 U/min respectively. The Km values were 34.6 g/L and 14.8 g/L respectively for the whole cells and supernatant. The results indicated that cellulosome secreted into supernatant had a prevailing maximum reaction rate as compared to that of cellulosome anchoring onto the cell walls. As to the Km value, it shows the supernatant cellulosome exhibits a higher substrate affinity than that anchoring into cell walls.

IV. CONCLUSION

In this short communication, a premilinary study about the use of the recombinant anchoring cellulosome from *B. subtilis* W800N strain was employed to degrade the Chlorella LDRs. There is about 2.4 U/mL of cellulosome activity secreted into the fermentation broth as compared to that anchoring in the Bacillus cell walls. The cellulosome activity was found mainly in the supernatant of the cultivation broth. The kinetics parameters of V_{max} and K_m values also display a prevailing effect for the cellulosome obtained in the supernatant to the whole cells. The kinetics data obtained would be quite helpful to the industrial application for the treatment of waste microalgae residuals.

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