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Communication

Enzyme-Assisted Extraction of Oil from Wet Microalgae *Scenedesmus* sp. G4

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Abstract: The enzyme-assisted aqueous extraction of oil from wet microalgae was employed to avoid the energy consumption of a dewatering process. In this paper, oil-rich microalgae *Scenedesmus* sp. G4 was hydrolyzed by enzyme mixtures for oil extraction. The results showed that the algae concentration had the greatest influence on yield of extracted oil, and the temperature and the ratio of enzyme mixtures affected the results as follows: the maximum yield of oil extracted from *Scenedesmus* sp. G4 reached 86.1% under optimal conditions with an algae concentration at 2.5 g/L, temperature 30/50 °C, pH 3.5/4.5 and a cellulase:pectinase:hemicellulase ratio of 1:1:1 or 1:2:1 (w/w/w). The results also indicated that the enzyme mixtures had a significant impact on the integrity of microalgae cells and the crystallization index increased from 30.7% to around 36.0% after enzymatic hydrolysis treatment. The cell wall of *Scenedesmus* sp. G4 has a high content of cellulose Iα and low crystallization, which is beneficial to the oil extraction by the enzyme-assisted hydrolysis method.

Keywords: *Scenedesmus* sp.; wet microalgae; oil extraction; enzyme-assisted hydrolysis

1. Introduction

Oil-rich microalgae are fast-growing organisms, able to fix large amounts of CO_2 and adapt to various environments. Their development and utilization are regarded as an important measure to contain global warming and guarantee national energy security [1,2]. The cost of large-scale utilization of microalgae energy, however, remains high [3]. Conventional oil extraction methods require dry microorganisms, and one of the major challenges is that the energy costs of a dehydration process before oil extraction are relatively high. Moreover, the conventional methods use chemical solvents that display insufficient recovery of extracts and toxicity and their unrenewable petroleum origin is undesirable. Nowadays, green extraction has become part of the sustainable development and industrial strategy [4,5]. Besides green extraction methods like ultrasound or microwaves [6], as shown in Table 1, enzyme-assisted aqueous extraction does not require a drying process and reduces the use of organic reagents in the extraction process, thus improving the economic efficiency and environmental benefits of the utilization of microalgae energy [7]. Compared with traditional chemical processes, extraction of energy substances using enzymatics method will not affect the quality of value-added biomass, and the high value-added coproduction of bioactivators greatly improves its competitiveness to rival fossil fuel [8].

Table 1. Comparison with conventional extraction methods.

Item	Conventional extraction	Enzyme-assisted extraction
Dehydration process	Needed	No
Organic reagent	Excess needed	Small
Quality of value-added biomass	Affected	Unaffected

The structure of the cell wall of microalgae is closely related to the effect of enzymolysis. The characteristic specificity of enzymes causes great limitations in single enzyme enzymolysis processes, and the cell walls of microalgae could be more thoroughly and effectively degraded by combining several kinds of enzymes. However mixed enzymatic hydrolysis studies always focus on large terrestrial plants, and hardly ever on methods for treating microscopic plants like microalgae. Also few reports have described the coordinative mechanism of enzymolysis in enzyme-assisted aqueous extraction of oil-rich microalgae.

In order to obtain a higher oil extraction yield, in this paper the oil-rich wet microalgae *Scenedesmus* sp. G4 was used to extract oil by enzyme-assisted hydrolysis. According to the orthogonal experiments, different extraction conditions were compared such as the ratio of cellulase, pectinase and hemicellulase, and the effects of operational parameters (*i.e.*, pH, temperature and microalgae concentration) on the yield of oil extraction were also investigated. The microalgae enzyme-assisted extraction mechanism was also initially analyzed by using modern methods such as scanning electron microscope (SEM), Fourier transform infrared spectroscopy (FT-IR), and X-ray diffraction (XRD).

2. Materials and Methods

2.1. Microalgal Strain and Culture Conditions

The freshwater green algae *Scenedesmus* sp. G4 was preserved at the Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences (Guangzhou, Guangdong, China). For the cultivation of this strain, BG11 medium [9] was used. About 100 mL of the pre-culture broths mentioned above were inoculated into a vertical tubular photobioreactor containing 1.0 L medium. The vertical tubular photobioreactor consisted of glass tubes of 70.0 cm height and 5.0 cm outside diameter. Light was supplied by white cool fluorescent lamps (Hengxing-T4, 24W) on one side of the photobioreactor (light intensity: 200 ± 50 μE/(m^2 s)). Aeration and mixing were achieved by compressed air enriched with 6.0% CO_2 through a glass-filter, which was inserted in the bottom of the reactor and the flow rate of the gas was 0.5 vvm regulated by a gas flow meter (Model G, Aalborg Instruments & Controls, Inc., Orangeburg, NY, USA). The temperature of the culture media was 25 °C ± 1 °C regulated by the room air conditioner (Gree Electric Appliances Inc., Zhuhai, Guangdong, China). A photo of the vertical tubular photobioreactors is shown in Figure 1. After 7 days of cultivation, the cultures were used for oil extraction by enzyme mixture hydrolysis.

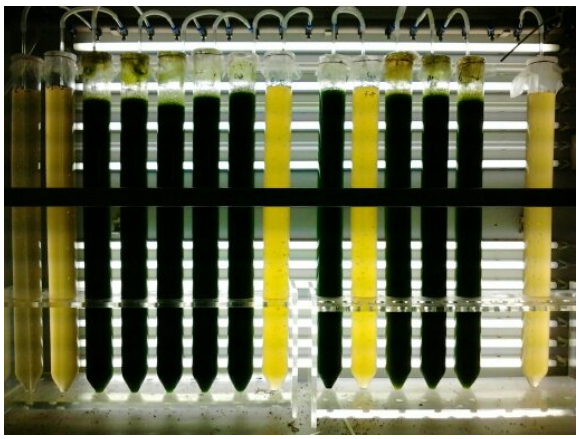

Figure 1. Photo of the vertical tubular photobioreactors.

2.2. Measurement Methods of Oil, Protein and Sugar Content

Total oil, protein and sugar content were measured according to Bigogno *et al.*'s method [10], Chu *et al.*'s method [11] and Yemm and Willis's method [12], respectively. The contents of oil, protein and sugar in dry weight (g/g dry weight) of *Scenedesmus* sp. G4 were 48.8%, 21.8% and 24.1%, respectively.

2.3. Enzyme Mixtures

Cellulase was purchased from Heshibi Biotechnology Co., Ltd. (Yinchuan, Ningxia, China) which was extracted from the fermentation liquor of *Penicillium* sp. cultures. Enzyme protein accounted for 10.08% (g/g dry weight). Pectinase and hemicellulase, both obtained from Yuanye Biotechnology Co., Ltd. (Shanghai, China) were extracted from the fermention of *Aspergillus niger* cultures. Protein accounted for 8.70% and 4.17% (g/g dry weight), respectively, in these substances.

2.4. Enzyme Assays

The filter paper enzyme activity of cellulase was measured according to method of the Commission on Biotechnology of the International Union of Pure and Applied Chemistry (IUPAC) [13]. Pectinase activity was evaluated by the method described by Meneghel *et al.* [14]. Hemicellulase activity was analyzed by xylanase activity measured according to Diogo *et al.*'s method [15]. The filter paper enzyme activity of cellulase was 7.2 U/mg protein. The enzyme activities of pectinase and hemicellulase were 500 U/mg protein and 1.5 U/mg protein, respectively.

2.5. Measurement of Oil Extraction

The enzymatic hydrolysis was performed in a 100 mL conical flask containing 30 mL of buffer solution. The enzymatic treatment was incubated in a water-bath shaker at 150 rpm for 72 h. The enzyme concentration was 2.0%. After enzymatic hydrolysis treatment, 20 mL hexane was added to each sample. The suspension obtained was mixed by a vortex mixer. Thereafter, the suspension was centrifuged at 5000 rpm for 5 min at 25°C. The supernatant was transferred to an evaporation flask. The solvent of the supernatant was eliminated by a vacuum rotary evaporator set at 30°C. The residue was re-extracted twice within 30 min. The flask was cooled and weighed to determine the oil content [7]. During the oil extraction process, 0.01% butylated hydroxytoluene (BHT) was added as antioxidant.

2.6. Orthogonal Array Testing of Enzyme Mixtures Hydrolysis

The orthogonal parameters were designed as shown in Table 2. Sodium azide, accounting for 0.03%, was added. In order to find the optimum condition of oil extraction, intuitive analysis was used.

Table 2. Levels and factors of orthogonal experiment for enzymatic hydrolysis.

Factors		Algae concentration (g/L)	Temperature (°C)	pH	Cellulase:pectinase:hemicellulase (dry weight ratio of protein)
Level	1	2.5	30	3.5	1:1:1
	2	5.0	40	4.0	2:1:1
	3	7.5	50	4.5	1:2:1
	4	10.0	60	5.0	1:1:2

2.7. Scanning Electron Microscopy

In order to observe the variations on the surface morphologies of microalgae after alkaline flocculation, these samples were examined using a high-resolution SEM (Model No.: S-3700N; Hitachi Co., Tokyo, Japan), which was operated at 10–20 kV accelerating potential. Prior to the observation, the surface of the sample was coated with a thin, electric conductive gold film.

2.8. Fourier Transform Infrared Spectroscopy Analysis

Infra-red spectra (IR) of the freeze-dried microalgae were measured on a KBr disk with a Tensor 27 FT-IR (Bruker, Billerica, MA, USA) to determine the functional groups of the microalgae cell surface. Absorbance spectra were collected at wavenumbers between 4500 cm^{-1} and 400 cm^{-1} with a nominal

resolution of 2 cm^{-1} with 64 scans collected for data acquisition. An empty well served as the background reference for each microplate.

2.9. X-Ray Diffraction Analysis

XRD measurements of the freeze-dried algal powder before and after alkaline flocculation were carried out on X'Pert Pro-MPD diffractometer (Panalytical, Almelo, The Netherlands) using the planar monochromator method and Cu Kα radiation (wavelength of 0.15406 nm). The samples were placed on a silicon holder. XRD data were collected in the range of 5°–80°, a step length of 0.017°, the radioactive rays under a voltage of 40 kV and an electric current of 40 mA. The crystallization index (*CrI*, %) will be calculated with equation [16,17]:

$$CrI=\frac{I_{main}-I_{am}}{I_{main}}\times 100$$

I_{main} is the diffraction intensity of the main peak (2θ = 20.1°) and I_{am} is the diffraction intensity of base line (2θ = 15.3°).

3. Results and Discussion

3.1. Results of Orthogonal Array Testing on the Enzymatic Hydrolysis

Pectinase, cellulase and hemicellulase were chosen as components of the enzyme mixtures. Compared with single enzymes, enzyme mixtures could further enlarge the channels of algae cell walls, to easily separate the oil molecules from the cells. Table 3 shows that different parameters had very different effects on the oil extraction yield. The algae concentration had the greatest influence on the yield of oil extraction, followed by the temperature and the ratio of the enzyme mixtures. The pH had the least influence on the oil extraction process. According to the test results shown in Table 4, the maximum yield of extraction oil reached 86.1% under the optimal conditions of 2.5 g/L algae concentration, 30/50 °C temperature, pH 3.5/4.5, and a cellulase:pectinase:hemicellulase ratio of 1:1:1 (w/w/w) or 1:2:1 (w/w/w).

Table 3. Analysis of variance (ANOVA) for the orthogonal test.

Factor	Sum of squares	Degree of freedom	*F*-value	Significance
Algae concentration (g/L)	7907.023	3	11.336	*
Temperature (°C)	619.913	3	0.889	-
pH	2.693	3	0.004	-
Cellulase:pectinase:hemicellulase (dry weight ratio of protein)	200.723	3	0.288	-
Pure error	697.53	3	-	-

Table 4. Results of orthogonal tests on the yield of oil extraction from *Scenedesmus* sp. G4 by enzyme-assisted hydrolysis.

No.	Algae concentration (g/L)	Temperature (°C)	pH	Cellulase:pectinase:hemicellulase (dry weight ratio of protein)	Yield (%)
1	2.5	30	3.5	1:1:1	86.1
2	2.5	40	4.0	2:1:1	61.5
3	2.5	50	4.5	1:2:1	86.1
4	2.5	60	5.0	1:1:2	69.7
5	5.0	30	4.0	1:2:1	41.0
6	5.0	40	3.5	1:1:2	30.7
7	5.0	50	5.0	1:1:1	51.2
8	5.0	60	4.5	2:1:1	34.8
9	7.5	30	4.5	1:1:2	23.2
10	7.5	40	5.0	1:2:1	20.5
11	7.5	50	3.5	2:1:1	27.3
12	7.5	60	4.0	1:1:1	28.7
13	10	30	5.0	2:1:1	16.4
14	10	40	4.5	1:1:1	12.3
15	10	50	4.0	1:1:2	28.7
16	10	60	3.5	1:2:1	16.4
I	75.850	41.670	40.125	44.575	-
II	39.425	31.250	39.975	35.000	-
III	24.925	48.325	39.100	41.000	-
IV	18.450	37.400	39.450	38.075	-
R	57.400	17.075	1.025	9.575	-

I, II, III, IV—sum of coverage rate of Levels 1–4, respectively. R: the difference between the largest sum value and the smallest sum value of each factor.

3.2. Scanning Electron Microscope Analysis of the Enzymatic Hydrolysis Products

As Figure 2 shows, before enzymatic hydrolysis *Scenedesmus* sp. G4 cells presented a smooth surface, the cellular morphology was clear and the cell walls complete.

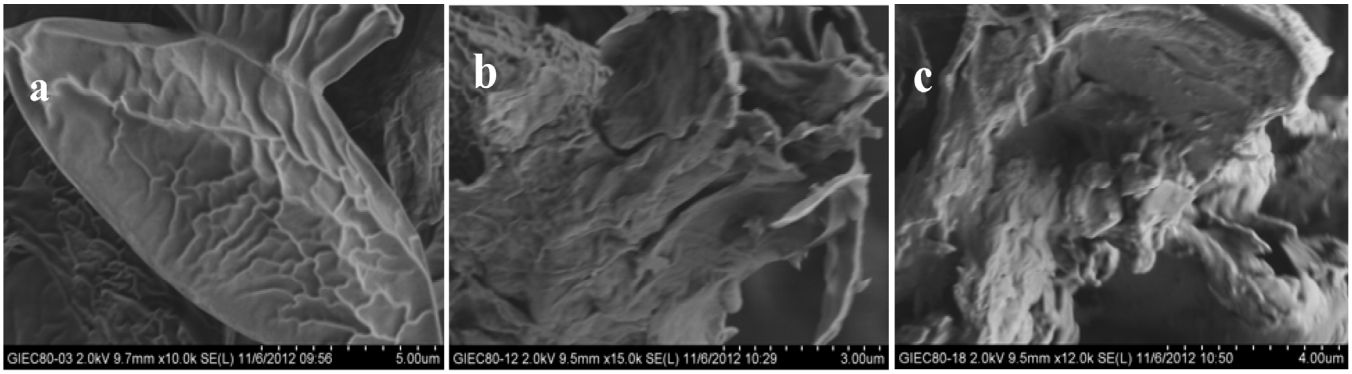

Figure 2. Scanning electron microscope (SEM) micrographs of *Scenedesmus* sp. G4 cultures: (**a**) before enzymatic hydrolysis; (**b**) hydrolyzed by enzyme mixtures 60 h, pH = 3.5; and (**c**) hydrolyzed by enzyme mixtures 60 h, pH = 4.5.

After hydrolysis by enzyme mixtures, the cell surface was rough and significant cell lysis occurred. The enzyme mixtures significantly damaged the intactness of microalgae cells, destroying the cell wall structure which was good for the next step of oil extraction.

3.3. The X-ray Diffraction Analysis of the Enzymatic Hydrolysis Products

As shown in Figure 3, the changes in the microalgae *Scenedesmus* sp. G4 after enzymatic hydrolysis were not significant according to the XRD results. The characteristic peaks at the positions of diffraction angles at 14.7°, 16.8°, 20.5° and 22.7° were attributed to the diffraction planes of 101, $10\bar{1}$, 021 and 002 in cellulose I. Since the composition of green algae basically do not contain lignin, and the content of cellulose Iα was relatively high, while typical lignocellulosic biomass has a relatively higher content of cellulose Iβ [18–20], the hydrogen bond in molecules of cellulose Iα was relatively weak, making the enzymolysis of endoglucanase easy [21] to facilitate the next step of oil extraction process. The crystallization index of *Scenedesmus* sp. G4 was increased after enzymatic hydrolysis. Figure 3 also shows that the crystallization index of *Scenedesmus* sp. G4 changes from 30.7% before enzyme hydrolysis to 36.0% after enzymatic hydrolysis.

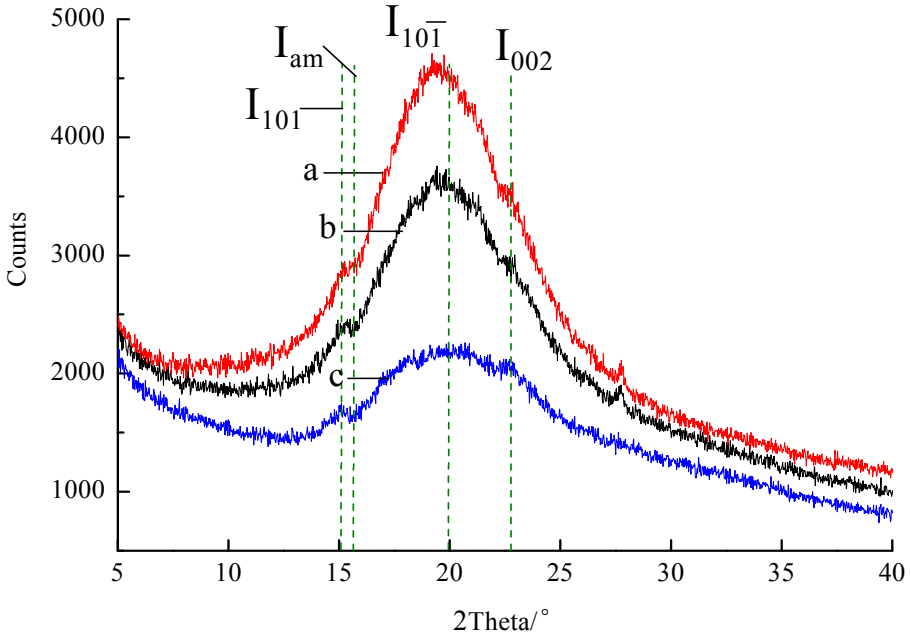

Figure 3. X-ray diffraction (XRD) patterns of *Scenedesmus* sp. G4 cultures: (**a**) before enzyme hydrolysis; (**b**) hydrolyzed by enzyme mixtures 60 h, pH = 3.5; and (**c**) hydrolyzed by enzyme mixtures 60 h, pH = 4.5.

3.4. The Fourier Transform Infrared Spectroscopy Analysis of Enzymatic Hydrolysis

The FT-IR spectra of the algae *Scenedesmus* sp. G4 before and after enzymatic hydrolysis are shown in Figure 4 [22,23]. The main component of the cell walls of green algae is cellulose, which is a glucose polymer connected by β-D-,4-glucosidic bonds. As shown in Figure 4 the broad peak at 3500–3300 cm^{-1} was attributed to –OH under the atomic force of H and O or –NH stretching vibrations. The carboxyl group absorption was located in the range of 2400–3300 cm^{-1}. The small shoulder at 2929 cm^{-1}

might be the vibration peak of associated carboxyl/phenolic groups. Compared with the microalgae *Scenedesmus* sp. G4 before enzymatic hydrolysis, the absorptions at 2929 cm^{-1}, 2855 cm^{-1}, 1743 cm^{-1}, 1158 cm^{-1} were increased after enzymatic hydrolysis. The cellulose and hemicellulose were exposed by the enzymatic hydrolysis. The cell wall bonds were changed by the mixed enzymatic hydrolysis, which improved the oil extraction significantly.

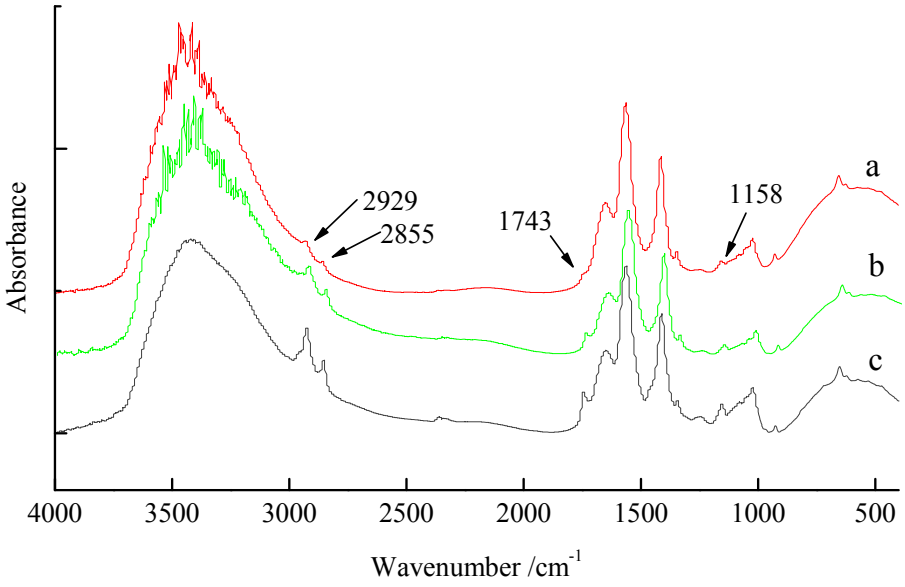

Figure 4. The changes of organic functional groups on *Scenedesmus* sp. G4 cultures: (**a**) before enzyme hydrolysis; (**b**) hydrolyzed by enzyme mixtures 60 h, pH = 4.5; and (**c**) hydrolyzed by enzyme mixtures 60 h, pH = 3.5.

4. Conclusions

Experimental results showed that oil extraction from wet microalgae can be directly carried out by enzyme-assisted extraction. The algae concentration had the greatest influence on oil extraction yield, followed by the temperature and the ratio of enzyme mixtures. The enzyme mixtures had a significant impact on the integrity of microalgae cells and the crystallization index was increased from 30.7% to around 36.0% after enzymatic hydrolysis. The cell wall of *Scenedesmus* sp. G4 with its high content of cellulose Iα and low crystallization index, was beneficial to the oil extraction by the enzyme-assisted hydrolysis method. The enzyme-assisted extraction of oil from wet microalgae could be a valid alternative to the conventional ways.

Acknowledgments

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Author Contributions

Shuhao Huo wrote the main part of the paper and performed the experiments. Zhongming Wang conceived the experiments. Fengjie Cui and Bin Zou revised the paper. Pengxiang Zhao and Zhenhong Yuan read and approved the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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