Enhancement of Lipid Production by *Euglena gracilis* Using Vanillin as a Growth Stimulant

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*Euglena gracilis* which could produce valuable metabolites is considered as promising feedstock for various industrial applications. Growth stimulation of microalgae culture with additives are cost-effective and simple approach to improve its productivity. Effect of vanillin, one of the most abundant phenolic compounds from lignocellulosic hydrolysate to *E. gracilis* was investigated. Vanillin showed hormesis effect to *E. gracilis*, growth promotion at lower concentration but inhibition at higher concentration. At optimal dosage of 10 mg/L vanillin, the biomass production of *E. gracilis* was enhanced by 36.5% and metabolites content such as chlorophyll, carotenoids also increased. From the high throughput analysis using fourier transform infrared spectroscopy, total lipid production will be simultaneously enhanced without sacrificing cell growth.

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**Key Words**

*Euglena gracilis*, Vanillin, Cell growth, Fourier transform infrared spectroscopy, Lipids

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**1. Introduction**

Sustainable feedstock which meets the demand of increasing human population and mitigate climate change are one of the global issue. Microalgae with single cells or simple multicellular bodies have been expected as a potential feedstock for food, animal feed, healthcare, and energy since a large number of active compounds can be produced by photosynthesis without conflict of land with food crops. The metabolites with attractive properties including pigments, proteins, polysaccharides, and fatty acids could be produced by microalgae. The unicellular flagellate alga, *Euglena gracilis*, can grow under autotrophic, heterotrophic, or mixotrophic conditions and contains a relatively large amount of valuable metabolites, such as vitamin, α-tocopherol, pigments, etc. Due to its rich metabolites and a wide range of applications, the large-scale production of *Euglena* is developed. However, low yield and high cost for *E. gracilis* culture still remained challenge to improve feasibility.

Enhancing the productivity is critical for economic and effective utilization of *E. gracilis* biomass. Genetic recombination engineering, and co-cultivations of microorganisms and microalgae have been reported. As a cost-effective alternative to increase biomass, supplementation with additives was considered to be promising. In previous research, 500 mg/L of ferulic acid from rice bran boosted the growth of *E. gracilis* up to 3.6 folds, 20 g/L of corn steep liquor and 20 mg/L of glucose was able to accumulate the lipid in algae *E. gracilis* up to 2.5 times. These earlier findings provided a new strategy to enhance microalgae biomass and metabolites using additives...
at low cost and high efficiency such as waste or unutilized resources.

Lignocellulosic compounds, which represent more than 90% of all plant biomass, are a kind of abundant renewable and sustainable resources. However, pretreatment is required to break the crosslinking of lignin, hemicellulose, and cellulose for further utilization. Though the profile of by-products mainly depend on the pretreatment methods, phenolic monomers from lignin decomposition are common compounds. Using these kinds of compounds as potential feedstock for microalgae cultivation may greatly decrease the cost of production. However, these kinds of phenolics are not all fully utilized by microalgae since they are considered to exert inhibitory activity against the growth of microorganism.

Vanillin (4-hydroxy-3-methoxybenzaldehyde), a low-molecular-mass guaiaeryl phenol, is one of the most abundant phenolic substances from the lignocellulosic. Vanillin is a natural resource that can be used in various industries. For example, it is widely used as a flavor material in food, beverages, perfume, and several other applications. The practical application of such kind of wastes is not achieved yet in microalgae cultivation due to the presence of less tolerance of some bacteria and fungi to vanillin. Unlike these bacteria and fungi, microalgae have stronger survivability. They can live in various extreme environments, such as high salinity, various pollutants, and other adverse environments. It will be attractive for microalgae culture if the vanillin promotes the growth.

Effect of vanillin on growth and lipid accumulation has not yet been investigated so far. Thus, the present work aimed to investigate the effects of vanillin to E. gracilis on growth, pigments, and biochemical components. Intracellular metabolites were evaluated by high-throughput Fourier transform infrared spectroscopy (FT-IR).

2. Materials and Methods

2.1 Microorganism and culture conditions

The unicellular freshwater algae, Euglena gracilis Klebs strain (NIES-48) was obtained from National Institute for Environmental Studies (Japan). Modified Cramer–Myers (CM) medium with the following composition (mg/L): (NH₄)₂HPO₄, 1000; KH₂PO₄, 1000; MgSO₄·7H₂O, 200; CaCl₂·2H₂O, 20; FeSO₄·7H₂O, 3; MnCl₂·4H₂O, 1.8; CoSO₄·7H₂O, 1.5; ZnSO₄·7H₂O, 0.4; Na₂MoO₄·2H₂O, 0.2; CuSO₄·5H₂O, 0.02; Vitamin B12, 0.0005; Thiamine HCl, 0.1 were used for culture. The CM medium, except the organic substrates, was sterilized by autoclave at 121 °C for 10 h, then move into desiccator and cooled to room temperature and the cell dry weight was calculated by following equation (1):

\[ \text{CDW} = (S_2 - S_1)/V \]

where \( S_1 \) (g) is the initial weight of filter and \( S_2 \) (g) is summation of the filter paper with dried biomass, respectively. \( V \) (L) is the volume of cell suspension.

Specific growth rates (r, d⁻¹) and doubling time (DT, d) were calculated for each group according to the following Eqs. (2) and (3):

\[ r = \ln N_2 - \ln N_1 \frac{t_2 - t_1}{t_1} \]

\[ \text{DT} = \ln 2 \frac{t_2 - t_1}{r} \]

where \( t_1 \) and \( t_2 \) denote beginning and end times of the log growth phase, respectively and \( N_i \) and \( N_f \) are cell densities (cells mL⁻¹) at times \( t_i \) and \( t_f \), respectively.

2.2 Growth measurement

The growth of E. gracilis was determined by cell density, optical density and dry weight, respectively. Cell number was measured by a counting chamber (Hirschmann, Thoma, Germany). In addition, pH of the culture filtrates was measured by pH meter (LAQUA-2103AL, Horiba, Japan).

Cell dry weight (CDW, g/L) of each group was also measured during the stationary growth phase. Aliquots of cell suspension were collected by centrifugation at 5000 rpm for 20 min, and harvested cell pellets were washed three times by distilled water to remove residues. Then, cells on the filter paper were transferred to oven (AVO-250 N, As one, Japan) at 80 °C for 10 h, then move into desiccator and cooled to room temperature and the cell dry weight was calculated by following equation (1):

\[ \text{CDW} = (S_2 - S_1)/V \]

Cell morphology was quantified through particle analysis under 12 h light/dark cycle conditions with white light at 5000 lx light intensity. The Flasks were manually shaken three times per day to ensure the light provided equally to cells and avoid its coagulation.

2.3 Cell morphology analysis

Cell aspect ratio (AR) is an essential parameter to analyze the growth of E. gracilis. Images of 100 cells were recorded using an optical microscope (Motic, BA210, Japan). Cell morphology was quantified through particle analysis with image processing software Image J (open source). Cell...
length was measured by the longest distance between any two points along the selection boundary and cell width was defined as the minimum caliper diameter. AR is expressed in the ratio of length to width.

2.4 Photosynthetic pigments analysis

The photosynthetic pigments including chlorophyll a, chlorophyll b and carotenoids were measured following the method of Lichtenthaler and Wellburn [21]. Aliquot 5 mL of algae were filtrated, and collected cells were washed with distilled water and then grinded with 80% acetone solution until cells were thoroughly crushed and the pigments were basically transferred into acetone. Afterwards, the extract homogenate was filtered again then collected and made up to 10 mL by 80% acetone in volumetric flask. Photosynthetic pigments content was determined by spectrophotometry. The absorbance of extract was measured at the wavelengths of 470, 646, and 663 nm. Pigments concentrations were calculated via Eqs. (4), (5) and (6):

\[
Chl_a = 12.21Abs_{663} - 2.81Abs_{646}
\]

\[
Chl_b = 20.13Abs_{663} - 5.03Abs_{646}
\]

\[
C_{cv} = \frac{(1000Abs_{663} - 3.27Chl_a - 104Chl_b)}{229}
\]

where \(Chl_a\), \(Chl_b\), \(C_{cv}\), denote the chlorophyll a, chlorophyll b and carotenoids concentrations, respectively. \(Abs_{663}\), \(Abs_{646}\), and \(Abs_{663}\) represent the absorbance of wavelength at 470, 646, and 663 nm, respectively.

2.5 Cellular component measured by FT-IR

FT-IR analysis was performed to determine the changes in intracellular macromolecules such as carbohydrates and lipids. Cells specimens were harvested by centrifugation at 5000 rpm for 20 min, and then re-suspended with deionized water and centrifuged again to eliminate the influence of vanillin and residual mineral salts in medium. The washed samples were frozen with liquid nitrogen and then transferred to vacuum freeze dryer, immediately (FDU-100, EYELA, Japan) at -50 °C for 24 h. Afterwards, freeze-dried biomass was equally mixed with potassium bromide (KBr) powder in the 1:100 ratio and then was pressed into flake by using hand press. The FT-IR spectrometer (Nicolet iZ10, Thermo Fisher Scientific, USA) and the corresponding spectrum software (OMINIC, Thermo Fisher Scientific, USA) were used to record and analyse samples. The infrared absorbance spectra of samples were obtained at 4 cm\(^{-1}\) resolution in range of 4000–400 cm\(^{-1}\) with 64 scans. Spectra were collected for triplicate per sample, resulting in 9 spectra at every concentration group. Spectrum of KBr pellet was collected as background. According to the previous research [22], all obtained spectra of samples were normalized to the amide I band at 1655 cm\(^{-1}\) to minimize the effect of sample thickness. Relative content of carbohydrate and lipids were acquired by analyzing carbohydrate/amide I and lipid/amide I peak height ratio.

2.6 Statistical analysis

Experiments were conducted in triplicate. Date were expressed as mean ± standard deviation. Statistical difference in the growth and metabolic parameters at different concentration treatment groups were determined by one-way analysis of variance (ANOVA), and student’s t-test was used for pairwise comparisons in which \(p\)-Value < 0.05 was accepted as statistically significant.

3. Results and discussion

3.1 Effect of vanillin on the growth profiles of E. gracilis

The growth of E. gracilis was promoted at vanillin concentration of 10 mg/L, whereas inhibited at higher concentrations over 100 mg/L (Fig. 1 (a)). At the end of cultivation, the E. gracilis cells dry weight at 10 mg/L of vanillin increased by 36.5% compared with the control (Fig. 1 (b)). It is worth noting that the growth promotion effect turned into inhibitory effect with vanillin concentration increased. CDW decreased to 38% and 48%, respectively at the 100 mg/L and 300 mg/L, then was completely inhibited at 500 mg/L. As shown in Fig. 1 (c), pH was within the range of 6-7 during culture, indicating that vanillin had little effect on the pH of the medium.

The specific growth rate and doubling time also supported for this kind of hormesis effect. As summarized in Table 1, the microalgal specific growth rate of 0.1 d\(^{-1}\) and doubling time of 7.12 d obtained at 10 mg/L vanillin was 1.05 and 1.40 times higher than the control, respectively. But the microalgae exposed to higher vanillin concentration over 100 mg/L, growth kinetics were worse than control group. These above results indicated that 10 mg/L of vanillin could improve the microalgae growth and could bear to some extent until vanillin concentrations around 300 mg/L, while hardly survive at the medium with 500 mg/L of vanillin.

The effect of vanillin on the microalgae growth were reported to be controversial in the previous studies. For instance, 40 mg/L of vanillin had obvious inhibitory effect on the growth of Alexandrium tamarense [23], the mortality of Microcystis aeruginosa Kützing was 50% with vanillin at 50 mg/L treatment [24]. The toxic effect of vanillin at higher concentrations might be related to the increase of phenolic monomers permeability to cell membrane leading to the leakage of cytoplasmic components and the influx of toxic compounds outside the cell [25]. However, it was reported that vanillin stimulated the growth of the red
Growth promotion were also observed with Dunaliella badawi-UTEX2538 and D. salina-UTEX200 at 25 to 50 mg/L vanillin treatment. The possible explanation for growth promotion might be because these algae species could metabolize vanillin as a carbon source. Vanillyl alcohol, which is less-toxic than vanillin was detected as a major metabolite of vanillin in Pycnoporus cinnabarinus and Rhodococcus opacus PD630. The positive effect of vanillin on some algae could be related to the capability of these algae to metabolize vanillin and use its metabolites with lower toxicity for their growth. Since the effects of phenolic compounds were known to be different depending on microalgal species, it is necessary to investigate the cell morphology and photosynthetic pigments to unveil the effect of vanillin to Euglena in this study.

### 3.2 Effect of vanillin on the morphological changes of E. gracilis

Cell shapes are considered as an indicator to reflect the cell status or physiology of E. gracilis. The cells exhibit spherical, spindle, and elongated-shape in response to different chemical or physical environments. Therefore, E. gracilis cellular morphology in this research was carefully investigated with microscope. Median cell width and length, and the cellular aspect ratio were presented in Fig. 2. Interestingly, the cell length increased and width decreased with the addition of 10 mg/L vanillin, and the aspect ratio was 1.24 times higher than that of the control group. In contrast, the decrease in cell length and the increase in the number of spherical shape cells was consistent with the emergence of the inhibitory effect at higher concentration (100, 300 mg/L) of vanillin treatment groups because the shape of this cyst is a protective mode of E. gracilis. It was reported that photosynthesis of E. gracilis would be enhanced when the cells change from spherical to elongated because the elongated shape is suitable for photosynthetic light capture.
electron transport \cite{30} and thus alter the rhythm of biological activities. The cell elongation observed at 10 mg/L of vanillin indicated that the promotion effect might be related to the improvement of photosynthesis of \textit{E. gracilis}.

### 3.3 Effect of vanillin on the photosynthetic pigments of \textit{E. gracilis}

Photosynthetic pigments content including chlorophyll \textit{a}, chlorophyll \textit{b}, and carotenoids in \textit{E. gracilis} cells under different concentrations of vanillin treatment were shown in Fig. 3. The chlorophyll \textit{a} content increased at vanillin concentration from 0 mg/L to 10 mg/L and then decreased with increased concentration of vanillin, which was consistent with cells growth profile. At the vanillin concentration of 10 mg/L, chlorophyll \textit{a} increased by 1.58 folds and then decreased at higher concentration. Chlorophyll \textit{b} slightly increased with vanillin concentration. However, carotenoids increased with the vanillin concentration under 100 mg/L while it decreased when vanillin concentration at 300 mg/L. The chlorophyll \textit{a}/\textit{b} ratio corresponded to the growth profile of \textit{E. gracilis}. Higher chlorophyll \textit{a}/\textit{b} ratio indicated better light absorbance capacity which resulted in growth promotion which clearly showed good accordance with Fig. 3 (b).

Similarly, some phenolic compounds, such as salicylic acid, protocatechuic acid are reported positive effects on the pigment content of \textit{Scenedesmus obliquus} \cite{32} and \textit{E. gracilis} \cite{33}. The regulatory impact of vanillin on the photosynthesis of \textit{E. gracilis} considered to be similar to that of some phytohormones as previously reported. All the photosynthetic pigments content of \textit{Chlorella sorokiniana SDEC-18} increased under phytohormones NAA (naphthylacetic acid) and IBA (indole-3-butyric acid) treatments \cite{34}. Some phytohormones, like IAA (3-indoleacetic acid), ABA (abscisic acid), EBL (24-epibrassinolide), and BL (brassinolide) were found to significantly increase chlorophyll

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**Fig. 2** Median cell length and width (μm) and aspect ratio of \textit{E. gracilis} cells cultivated under different vanillin concentration \((n = 100)\)

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**Fig. 3** (a) Photosynthetic pigments content (%CDW) under different concentrations of vanillin; (b) Ratio of chlorophyll \textit{a}/\textit{b}. Error bars represent the standard deviation \((n = 3)\) (ND=Not Determined)
concentrations in Scenedesmus quadricauda, which supports our hypothesis. Chlorophyll $a$ and chlorophyll $b$ play a significant role in capturing light energy, and high pigment content under vanillin treatments indicates the better efficacy of photosynthesis. Carotenoids are essential to the stability and function of the photosynthetic devices, not only used as photoprotectants to prevent harmful photodynamic reactions, but also used as auxiliary light-collecting pigments, extending the spectral range of light-driven photosynthesis. In addition, carotenoids can be produced from lipids and other basic organic metabolic building blocks by algae. Therefore, measurements of microalgae metabolites were necessary to better understand the physiological response of the *Euglena* to vanillin.

3.4 Cellular biochemical changes based on FTIR spectra analysis

FT-IR spectroscopy was applied to *E. gracilis* cells to analyze macromolecular composition with or without vanillin treatment (10 mg/L). Within the spectral range of 1800–1000 cm$^{-1}$, all macromolecules showed distinct absorption spectra as expected from band assignments presented in related literature. The important absorption band of vC=O-C appeared at 1074 cm$^{-1}$ was related to carbohydrate and the bands of vC=O and δN-H at 1655 and 1540 cm$^{-1}$ were related to amide I and amide II, respectively, corresponding to proteins. The absorption band of vC=O at 1734 cm$^{-1}$ was assigned to lipids. The main metabolites could be identified with the FT-IR spectra. Relative content of lipid and carbohydrate was calculated based on the ratio of the lipid band (1734 cm$^{-1}$) and carbohydrate band (1074 cm$^{-1}$) to the amide I band (1655 cm$^{-1}$), respectively. As shown in Fig. 4 (a), the absorption band of vC=O is more obvious in the 10 mg/L vanillin treatment group, while the absorption band of vC=O-C decreases compared to control, which represents the changes in lipid and carbohydrate content, respectively. Compared to the control group, relative lipid content increased to 3.17% and the relative carbohydrate content decreased to 8.48% with 10 mg/L vanillin treatment, respectively (Fig. 4 (b) and (c)). The results were consistent with the finding that hydroxybenzaldehyde and syringaldehyde increased lipid content by 6.81% and 5.39%

![Normalized FT-IR spectra (1800–800 cm$^{-1}$) of *E. gracilis* cultured with or without 10 mg/L vanillin; (b) Comparison of relative lipid and (c) carbohydrate content of *E. gracilis* cultured with or without vanillin. Error bars represent the standard deviation (n = 9)](image-url)
respective in *E. gracilis* 38).

Simultaneous lipid and biomass accumulation are considered to be the promotion effect of vanillin on the net photosynthetic rate, and the remaining carbon made by photosynthesis generated more acetyl-CoA through glycolysis, which can be converted to fatty acids 39). Fatty acids are initially synthesized in chloroplasts and then exported for further modification into lipids. In contrast, the pyruvate produced by glycolysis leads to carbohydrates, which could be reasonable explanation of our results in this study. In general, cell growth and lipids accumulation are considered to be opposites, and lipids accumulate in an adverse environment. The most common approach on lipids accumulation was to increase the content of lipids per unit of biomass cultured under nutrient limitation, such as nitrogen deficiency, phosphorus deficiency, and high-salinity stress, forcing cells to adjust biosynthetic pathways to accumulate lipids 40) ~ 43). These stress conditions initially improve the amount of lipid, but in long shot are not good strategy because they limit the overall growth and sacrificing biomass productivity during culture. Our finding provides very encouraging strategy for the commercial production of lipids by microalga which could increase both lipids and biomass productivity at the same time with the vanillin dosage at optimal concentration.

4. Conclusion

The effect of vanillin on the growth of *E. gracilis* was evaluated for the first time in this study. Vanillin at the optimal concentration of 10 mg/L promoted cell growth by 36.5%. Metabolites of *E. gracilis* such as chlorophyll, carotenoids were improved. Furthermore, total lipid production will also be enhanced due to the increase of biomass. While vanillin concentration increased from 10 mg/L to 100 mg/L, growth profile turned out to inhibitory effect. These results supported potential of vanillin as a growth stimulator for algal growth and lipid production because phenolic compounds including vanillin could be easily and economically obtained from hydrolysis of lignocellulosic materials.

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