The effect of vanillin on *Chlorella* growth

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Abstract

_in this work the effect of vanillin on the growth of Chlorella was evaluated. During a 6 - day cultivation, Chlorella growth was fully enhanced by vanillin at concentration of 50 mg/L. Vanillin concentrations: 25, 100, 150, 200 mg/L initially caused suppressing effect with further recovery of Chlorella growth. Vanillin at concentration of 300 mg/L fully inhibited Chlorella culture. The stimulating effect of vanillin (as for 25 and 50 mg/L) was indicated both by the increase of biomass density and chlorophyll content in Chlorella culture._

**Keywords:** microalgae, vanillin, biomass, chlorophyll

1. Introduction

4-hydroxy-3-methoxybenzaldehyde, commonly known as vanillin, is an organic compound possessing aldehydic, etheric and phenolic functional groups. The molecular formula of vanillin is C₈H₈O₃ with molecular weight equal to 153.15. Vanillin can be naturally found in bean pods of the tropical orchid *Vanilla planifolia* [1]. Worldwide, there is an industrial demand for vanillin as a flavouring agent, fragrance ingredient or pharmaceutical precursor [2]. Many methods were developed to produce vanillin: solvent extraction from cured *Vanilla* pods [3] (Figure 1 A), a synthetic condensation process between guaiacol and glyoxylic acid with further oxidative decarboxylation [4], alkaline oxidation of lignosulphonates obtained from wood (Figure 1 B) pulp mills [5] or enzymatic bioconversion of ferulic acid [6]. Vanillin also shows biological activity towards a wide branch of organisms including fish [7], aquatic invertebrates [8], earthworms [9], yeast [10] and algae [11].

![Figure 1. Production of vanillin from vanilla bean pods (A) or wood (B)](image-url)

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2. Materials and methods

2.1 Chlorella growth

*Chlorella* sp. was obtained from Culture Collection of Baltic Algae (CCBA). The strain was cultivated in a modified Bold’s Basal Medium (BBM). Medium composition was as follows: K$_2$HPO$_4$ (0.075 g/L), KH$_2$PO$_4$ (0.175 g/L), MgSO$_4$7H$_2$O (0.075 g/L), NaNO$_3$ (0.25 g/L), CaCl$_2$2H$_2$O (0.025 g/L), NaCl (0.025 g/L), Na$_2$EDTA2H$_2$O (0.025 g/L), FeCl$_3$ (6 mg/L), H$_3$BO$_3$ (1 mg/L), ZnSO$_4$7H$_2$O (1.4 mg/L), MnSO$_4$H$_2$O (0.16 mg/L), CuSO$_4$5H$_2$O (0.16 mg/L), CoCl$_2$6H$_2$O (0.065 mg/L), Na$_2$MoO$_4$2H$_2$O (0.135 mg/L). *Chlorella* was cultivated in 500 ml round flasks with an initial culture volume of 160 ml, in an incubator (Certomat® KT) equipped with a light source (fluorescents lamps, 5 X 18 W) and a shaker (110 rpm). The culture was incubated in day/night cycles (16h/8h) during the period of 6 days. Different concentration of vanillin: 0, 25, 50, 100, 150, 200, 300 mg/L were present in 160 ml growth media. Vanillin (POCH) was dissolved in media with no addition of organic solvents.

2.2 Biomass density measurement

Optical density of *Chlorella* biomass in media with different vanillin concentration was determined by taking aliquots of the culture and measuring absorbance (T80+ UV/VIS Spectrometer PG Instruments Ltd) at wavelength 530 nm (OD$_{530}$). Measurement days were: 0 (after inoculation), 2, 3, 4, 5, 6. There was no measurement after the first day of cultivation as it was time necessary for microalgae to adapt to new growth conditions. Optical density of the culture in the presence of vanillin was compared with the control culture to which no vanillin was added. Comparison was made between samples from the same particular day (X) and *Chlorella* growth was expressed as a percent of control.

\[
\mu_X = \frac{OD_{530v_X}}{OD_{530c_X}}
\]

Where:

- $\mu_X$: relative growth rate expressed as a percent of control at specific day (X) [%]
- OD$_{530v_X}$: optical density of culture in the presence of vanillin at specific day (X) [-]
- OD$_{530c_X}$: optical density of control culture at specific day (X) [-]

2.3 Chlorophyll extraction and measurement

During cultivation, samples of culture aliquots were taken and added to laboratory tubes and centrifugated to remove media. After removing media, pure methanol was added to tubes. Sealed tubes were shaken to ensure that microalgae cells are in the whole solvent volume. Extraction was carried out for a period that enabled the complete removal of green color from treated materials. When the extraction was complete, aliquots were centrifuged to separate cells from solvent because floating cells cause interferences during chlorophyll measurement. Chlorophyll $a$ and $b$ in obtained extracts were spectrophotometrically measured according to equations [12] presented below:

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Equations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol (pure)</td>
<td>$\text{Chl}<em>a = (16.72 A</em>{665} - 9.16 A_{652}) \cdot V_{\text{methanol}} / V_{\text{culture}}$</td>
</tr>
<tr>
<td></td>
<td>$\text{Chl}<em>b = (34.09 A</em>{652} - 15.28 A_{665}) \cdot V_{\text{methanol}} / V_{\text{culture}}$</td>
</tr>
</tbody>
</table>
Where:

- $\text{Chl}_a$: chlorophyll $a$ concentration in the culture [µg/ml]
- $\text{Chl}_b$: chlorophyll $b$ concentration in the culture [µg/ml]
- $A_{665}$: absorbance at wavelength 665 nm [-]
- $A_{652}$: absorbance at wavelength 652 nm [-]
- $V_{\text{methanol}}$: volume of methanol used for chlorophyll extraction [ml]
- $V_{\text{culture}}$: volume of culture used for chlorophyll extraction [ml]

2.4 Vanillin concentration measurement

Concentration of vanillin in culture media during a 0, 4 and 6 day of cultivation was evaluated by Total Phenol Analysis Method [13]. Culture samples from specific cultivation days were taken and centrifuged. Supernatant without microalgae culture was taken in the volume of 0.1 ml and mixed with 7.9 ml of distilled water and 0.5 ml of 2 N Folin – Ciocalteu reagent. After 8 minutes, 1.5 ml of 20 % Na$_2$CO$_3$ was added and shaken to mix. Reagent mixture was kept in a water bath at 40 °C for 30 minutes. The absorbance of solution was measured at the wavelength of 765 nm and vanillin concentration was determined from standard curve plotted for vanillin with the use of the same method.

3. Results

Results of present study show a varying effect of vanillin on *Chlorella* growth. During first days of cultivation, vanillin at concentration of 25, 100, 150, 200 and 300 mg/L (Figure 2 A & B) caused inhibition of microalgae growth and inhibition rate increased with higher amount of vanillin used. However next cultivation days showed substantial recovery of *Chlorella* growth. Microalgae culture with vanillin concentration of 25 mg/L and a slight inhibition effect at the beginning of cultivation showed (after 4 days) growth acceleration and the increase in biomass density higher than the control culture (no vanillin added). On the other hand, vanillin at concentration of 100 mg/L firstly caused stronger inhibition (after 2 days) than 25 mg/L of this phenolic compound, but then progressing recovery (2 – 3 days) occurred and final growth rate was higher than the culture with 25 mg/L vanillin and much higher when compared to control. Vanillin at higher concentration was found to exert stronger suppressing effect on *Chlorella*, with delayed recovery effect after 3 and 4 days, respectively for 150 and 200 mg/L and biomass density at the same level as control during the end of a 6 day cultivation. The highest concentration of vanillin (300 mg/L) resulted in the strongest inhibition and no growth recovery during *Chlorella* cultivation. Surprising results were obtained with 50 mg/L of vanillin in culture media. At this concentration no inhibition effect occurred. What is more, the last 3 days of cultivation were characterized by the highest increase in biomass density in relation to control from all tested concentration (Figure 2 A). The amount of microalgae inoculum added to fresh media before cultivation varied between performed experiments. It this research, biomass content at the onset of cultivation (OD of 0.048 and 0.088) did not change the effect of vanillin on *Chlorella* growth. Experiments with different biomass content showed the same enhancing effect of vanillin (25 and 50 mg/L) after a 6 day cultivation in both experiments (Figure 3 A & B). The acceleration of *Chlorella* growth in case of 25 or 50 mg/L vanillin was detected not only due to the increase in biomass density but also by chlorophyll content higher than in the control culture. Increased amount of chlorophyll $a + b$ content (expressed as % of control) was found both during a 4 and 6 day of cultivation (Table 1). Additionally, it was revealed that initial vanillin concentration of 50 mg/L decreased drastically during 4 days of cultivation with its complete removal from growth media after 6 days (Table 2).
Figure 2. The effect of vanillin at concentration of 25, 50 mg/L (A) and 100, 150, 200, 300 mg/L (B) on Chlorella growth expressed as % of control.
Figure 3. The influence of initial biomass content: $OD_{530}$ of 0.048 (A) and 0.088 (B) on Chlorella growth in the presence of vanillin at concentration of 0, 25 and 50 mg/L.

Table 1. The effect of vanillin at concentration of 25, 50 mg/L on chlorophyll $a + b$ concentration in Chlorella culture expressed as % of control.

<table>
<thead>
<tr>
<th>Cultivation day</th>
<th>Vanillin concentration [mg/L]</th>
<th>Chlorophyll a + b [% of control]</th>
<th>Chlorophyll a + b [% of control]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>108</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>119</td>
<td>125</td>
</tr>
</tbody>
</table>

Table 2. Concentration of vanillin in Chlorella culture during a 6 day cultivation.

<table>
<thead>
<tr>
<th>Cultivation day</th>
<th>0</th>
<th>4</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vanillin concentration [mg/L]</td>
<td>50</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>
4. Discussion
In literature, phenols are reported to possess inhibitory impact on microalgae. The decay of barley straw, rice straw, mugwort (Artemisia asiatica) and chrysanthemum showed inhibitory activity on growth of Microcystis sp. and Scenedesmus sp. It was suggested that ester and phenolic compounds released from decomposing plant materials were responsible for growth inhibition of tested algae [14]. Eurasian water milfoil (Myriophyllum spicatum) has been reported to release polyphenols inhibiting growth of Microcystis aeruginosa [15]. The essential oils obtained from rigid hornwort (Cerathophylum demersum) and eel grass (Vallisneria spiralis) exerted inhibitory effect on growth of Microcystin aeruginosa. The oils were composed of fatty acids, esters, terpenoids, phenolic compounds and phthalates [16]. However, in research mentioned above, inhibitory effect was attributed not only to phenols but also to other various compounds in the mixture. In another study, it was revealed that Chlorella vulgaris and Scenedesmus bijugatus were sensitive to phenol and nitrophenols in photoautotrophic cultures [17]. What is more, one research reported that vanillin at concentration of 152 mg/L caused 50 % inhibition of Chlorella vulgaris after 3.3 cultivation days and only 30 % inhibition after 6.6 days of cultivation [18]. It is partially comparable with results of present research as vanillin at 100, 150 and 200 mg/L concentrations initially caused suppressing effect with further alleviation of inhibitory activity towards Chlorella growth. However, nothing is known about the stimulating effect of vanillin on microalgae in photoautotrophic cultures. Such enhancing effect was detected in present study with vanillin at concentration 50 mg/L (full enhancement) and 25, 100 mg/L (initial inhibition and further enhancement). Stimulation of Chlorella growth at 50 mg/L of vanillin was accompanied by removal of vanillin from culture media. It was mentioned that Ankistrodesmus braunii and Scenedesmus quadricauda showed ability to degrade phenols with a removal above 70 % within 5 days [19]. Also, Clorella vulgaris was able to degrade 9.05 % of phenol after 7 days [20]. Results obtained from present research and literature data suggest that vanillin can be metabolized by microalgae. Additionally, the presence of vanillin in growth media can exert not only suppressing but also enhancing effect on microalgae culture.

5. Literature


