

# The effect of indomethacin on the growth and metabolism of green alga *Chlorella vulgaris* Beijerinck

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**Abstract** The aim of the present work was to study the effect of indomethacin (IM), a pleiotropic therapeutic substance commonly used in animal systems, at concentration range of  $10^{-8}$  to  $10^{-3}$  M on the growth and metabolism of single-celled *Chlorella vulgaris* (Chlorophyceae). Because of the presence of the indole ring in its molecule, IM is characterized by structural similarity with natural auxins, e.g. IAA. It was found that IM influenced algal growth, macromolecular synthesis and metabolism in dose-dependent manner. IM had the highest stimulating effect on algae at  $10^{-7}$  M on the 5th day of culture resulting in the increase in cell number and dry mass, DNA, RNA, proteins, phosphates, monosaccharides, photosynthetic pigments and glycolic acid content as well as protein extracellular secretion to the environment. Specific proteins from the region 20–139 kDa appeared during  $10^{-7}$  M IM treatment on the 5th day of cultivation as analysed by SDS-PAGE. IM-induced photosynthetic oxygen exchange in green alga was also noted. In contrast, the treatment with IM at the highest concentration of  $10^{-3}$  M suppressed cell division, dry mass production and decreased the level of the analysed parameters during the whole 7-day period of cultivation. Therefore, it could be

speculated that IM functioned as a plant growth regulator affecting cell division and metabolism of green alga *C. vulgaris*.

**Keywords** Dry mass · DNA · Glycolic acid · Monosaccharides · Phosphates · Photosynthesis · Photosynthetic pigments · Proteins · RNA

## Introduction

Auxins are the class of phytohormones involved in the numerous aspects of plant growth and development at the molecular and whole-plant level. Indole-3-acetic acid (IAA) is the natural auxin commonly occurring in all vascular plants and in green algae from genus *Chlorella* and *Scenedesmus* (Jacobs 1993; Stirk and Van Staden 1997; Mazur et al. 2001). Auxins regulate the rate of cell division, elongation and cell expansion, ethylene biosynthesis, root development, leaf formation, apical dominance and differentiation of vascular tissues (Petrásek et al. 2002; Woodward and Bartel 2005; Shinohara et al. 2006). Synthetic auxins: phenylacetic acid (PAA), 1-naphthalacetic acid (NAA) and popular herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) induce similar physiological responses in bioassays like natural auxins (Grossmann 2000; Czerpak et al. 2004; Imin et al. 2005).

Indomethacin (IM) includes an indole ring interlinked with a carboxyl group like natural auxin—IAA

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### Determination of photosynthetic pigments and photosynthetic oxygen exchange

The content of photosynthetic pigments followed homogenization of *C. vulgaris* fresh weight in 99.9% methanol. The absorbance of the extract was measured with a spectrophotometer at 652.4 and 665.2 nm for chlorophylls *a* and *b*, at 654.2 and 647.6 nm for phaeophytins *a* and *b*, at 470.0 nm for carotenoids. The amounts of photosynthetic pigments present in the methanol extract were calculated according to the equations of Wellburn (1994). Photosynthesis capacity was determined by measuring the amounts of oxygen released by the cells using a Clark type oxygen electrode. A 2-ml algal suspension was incubated in a vessel at 25°C and 1,000 mmol m<sup>-2</sup> s<sup>-1</sup> PAR. The evolution of oxygen in the medium was calculated to represent the activity of photosynthesis of *C. vulgaris*.

### Measurement of glycolic acid content

For glycolic acid extraction algal suspension were centrifuged at 14,000 rpm for 6 min at 4°C (Kehlenbeck et al. 1995). The supernatant was removed and the cell pellet was frozen overnight at -20°C. The next day the cell pellets were thawed at room temperature and extracted by addition of 5 mM HCl, followed by heating in a water bath at 60°C for 5 min. After heating, the cell extract was cooled and centrifuged for 5 min at 12,000 rpm, and the supernatant were assayed for glycolate by the Calkins method (Calkins 1943).

### Extraction of DNA

For DNA determination, the algae were first collected by centrifugation (4,500 rpm, 15 min, 20°C) of 10-ml culture samples and then the algal pellet was quickly frozen in liquid nitrogen, powdered with a mortar and pestle and transferred into extraction buffer (100 mM Tris-HCl, pH 8.0, 1.4 mM NaCl, 2% (w/v) cetyltrimethylammonium bromide (CTAB), 20 mM ethylenediaminetetraacetic acid (EDTANa4), 1% (w/v) polyvinylpyrrolidone (PVP) 40000, 1% (w/v)  $\beta$ -mercaptoethanol). The extract was slightly mixed and incubated in a water-bath at 65°C for 30 min. Then 10 ml chloroform-isoamylalcohol (24/1, v/v)

was added and centrifuged (10,000 rpm, 10 min, 20°C). The supernatant was transferred into a new tube, mixed with 2/3 volume isopropanol by inverting the tubes several times and incubated at 20°C for 30 min. After centrifugation (10,000 rpm, 15 min, 20°C), the supernatant was transferred into a new tube, mixed with 10 ml 76% (w/v) ethanol/10 mM ammonium acetate. After another centrifugation (10,000 rpm, 10 min, 20°C), the supernatant was discarded completely. The pellet was dried under vacuum, dissolved in 1 ml 1× TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and extracted with one volume 99% ethanol/7.5 mM ammonium acetate. Finally, the tubes were centrifuged again (9,500 rpm, 10 min, and 4°C), the pellet was dissolved in 1× TE (pH 8.0). The concentration of DNA was determined by measuring the optical density at 254 nm (OD<sub>254</sub>). A solution of DNA whose OD<sub>254</sub> = 1 contains approximately 50  $\mu$ g ml<sup>-1</sup> (Rogers and Bendich 1985).

### Extraction of RNA

Total RNA was extracted according to the protocol of Sambrook et al. (1989). For total RNA determination, the algae were first collected by centrifugation (4,500 rpm, 15 min, 20°C) of 10-ml culture samples, and the algal pellet was then quickly frozen in liquid nitrogen, powdered with a mortar and pestle and transferred into extraction buffer (0.05 M Tris-HCl, pH 9.0; 0.1 M NaCl; 0.1 M EDTA; 2% (w/v) SDS). The extract was gently mixed, 17.5 ml phenol and 17.5 ml chloroform/isoamyl alcohol (19:1, v/v) were added, and the mixture was briefly agitated and centrifuged (3,700g, 10 min, 20°C). The aqueous phase was then transferred into a new tube, re-extracted with the phenol/chloroform/isoamyl alcohol mixture, and centrifuged. The supernatant was carefully discarded, the pellet was re-dissolved in a small volume of water treated with diethyl pyrocarbonate, and an equal volume of 8 M LiCl was added. The pellet was stored at 4°C for 24 h, centrifuged, and the supernatant was carefully discarded. The pellet was washed twice with 70% ethanol, centrifuged briefly, and the supernatant was discarded. The pellet was then dried and resuspended in a small volume of water treated with diethyl pyrocarbonate. To determine the RNA concentration, the OD<sub>260</sub> was measured. A solution of RNA whose OD<sub>260</sub> = 1 contains approximately 40  $\mu$ g ml<sup>-1</sup>.

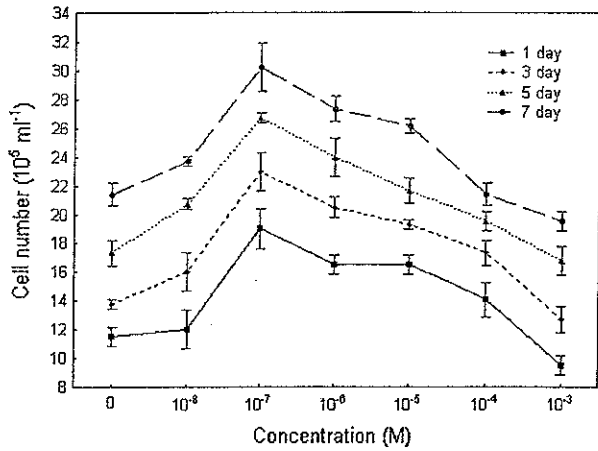


Fig. 2 The effect of 10<sup>-8</sup> to 10<sup>-3</sup> M IM on the cell number of *Chlorella vulgaris* compared to the control culture (0). Mean values ± SD, n = 6

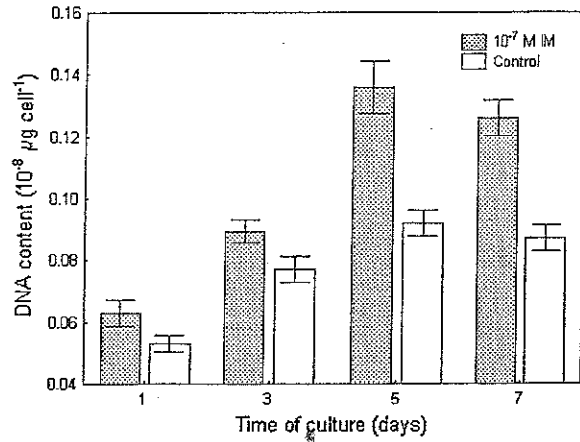


Fig. 4 The content of DNA in *Chlorella vulgaris* cells under the influence of 10<sup>-7</sup> M IM compared to the control. Mean values ± SD, n = 4

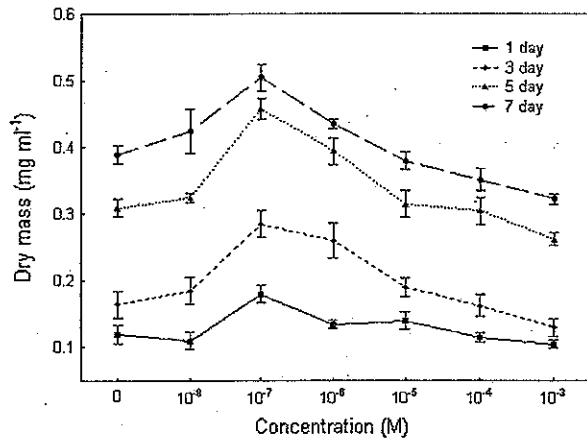


Fig. 3 The effect of 10<sup>-8</sup> to 10<sup>-3</sup> M IM on the dry mass of *Chlorella vulgaris* compared to the control culture (0). Mean values ± SD, n = 6

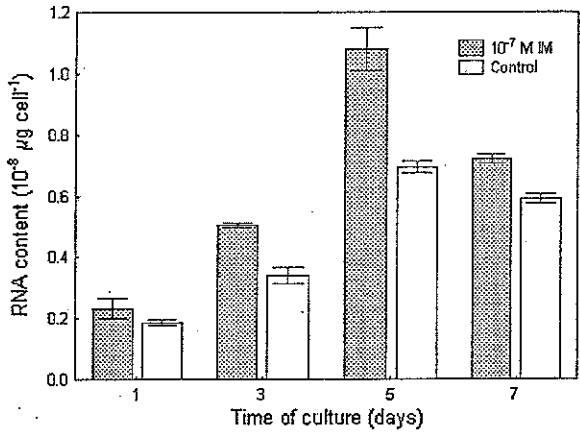
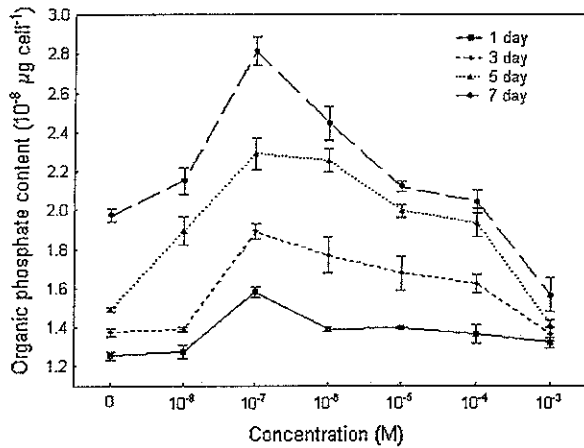


Fig. 5 The content of RNA in *Chlorella vulgaris* cells under the influence of 10<sup>-7</sup> M IM compared to the control. Mean values ± SD, n = 4

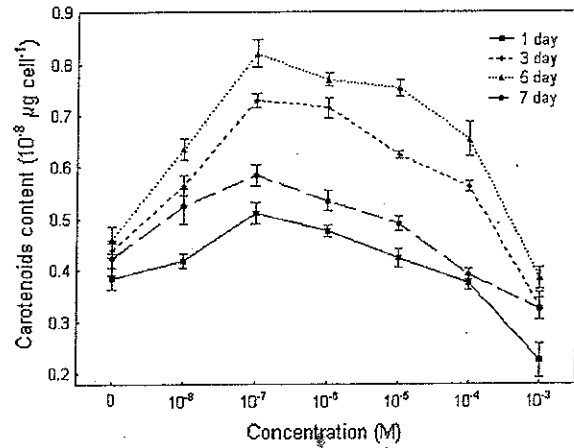
synthesis in *C. vulgaris* was performed (Figs. 4, 5). The obtained data showed that DNA level in IM-treated algal cells increased within the range of 44–48% between the 5th and 7th day of culture. Addition of 10<sup>-7</sup> M IM affected also higher by 52–60% accumulation of RNA above the control culture on the 5th and 7th day of the experiment.

Water-soluble proteins synthesis, their profile and secretion in response to 10<sup>-8</sup> to 10<sup>-3</sup> M IM was examined (Figs. 6–8). IM at extremely inhibitory dose on plant growth (10<sup>-3</sup> M) affected reduction of more than 20% in protein content. By contrast, lower concentrations of IM demonstrated stimulating influence on the protein production. The highest increase in the water-soluble protein content to 43% over the

control was shown on the 5th day of the experiment as a result of 10<sup>-7</sup> M IM application. Because 10<sup>-7</sup> M IM induced maximal protein production in algal cells on the 5th day of culture, SDS-PAGE of total proteins was performed. Five-day-old *C. vulgaris* cells grown with the presence of 10<sup>-7</sup> M IM accumulated approximately 24 polypeptides with molecular weight from 16 to 139 kDa in relation to the control (about 6 with Mr 16–56 kDa). Approximately 18 abundant polypeptides bands with molecular masses 20–139 kDa seemed to be specific response to IM treatment because they weren't detected in the control. Furthermore, polypeptide profiles of total cellular proteins showed significant concentrations of polypeptide bands with Mr 16, 20,



**Fig. 10** The content of organic phosphates in *Chlorella vulgaris* cells under the influence of 10<sup>-8</sup> to 10<sup>-3</sup> M IM compared to the control (0). Mean values ± SD, n = 6



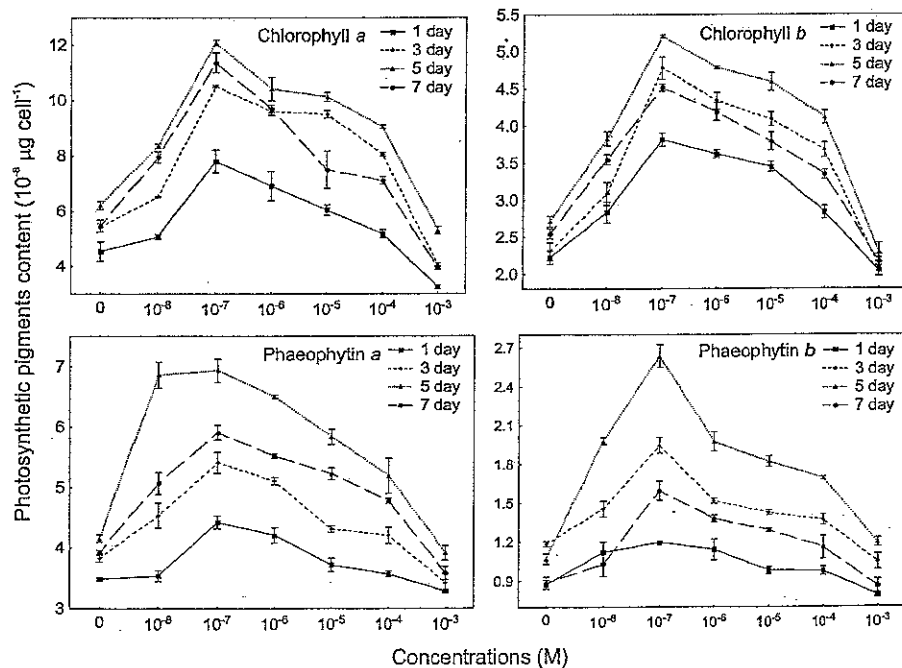
**Fig. 12** The content of carotenoids in *Chlorella vulgaris* cells under the influence of 10<sup>-8</sup> to 10<sup>-3</sup> M IM compared to the control (0). Mean values ± SD, n = 6

inorganic phosphates and on the 5th day in relation to organic phosphates in response to 10<sup>-7</sup> M IM. The application of 10<sup>-3</sup> M IM to cultured *C. vulgaris* cells had a negative effect on phosphorus nutrition and resulted in 10–20% decrease in organic and inorganic phosphates content from the 3rd day of the experiment.

Photosynthesis enhancement is an often observed feature in plant cells in response to auxins. For this reason changes in the production of pigments involved in photosynthesis were tested under the influence of 10<sup>-8</sup> to 10<sup>-3</sup> M IM (Figs. 11–13). The highest dose of IM (10<sup>-3</sup> M) contributed to

significant reduction by about 30% of chlorophyll *a*, 15% chlorophyll *b*, 40% carotenoids and 10% both phaeophytin *a* and *b* accumulation in relation to the control. On the other hand, cultivation of algae with the most active concentration of IM (10<sup>-7</sup> M) caused more than 90% increase in chlorophylls content and 70% in carotenoids level as well as great 70–140% increase in phaeophytins amounts in *C. vulgaris* cells. Photosynthetic oxygen evolution in algal cell suspension measured under the influence of the most active concentration 10<sup>-7</sup> M IM displayed close correlation with the enhanced photosynthetic pigment

**Fig. 11** The content of photosynthetic pigments in *Chlorella vulgaris* cells under the influence of 10<sup>-8</sup> to 10<sup>-3</sup> M IM compared to the control (0). Mean values ± SD, n = 6



Phytotoxic activity of the highest dose of IM ( $10^{-3}$  M) in *C. vulgaris* may be explained by IM-induced biosynthesis, conjugation and degradation which allow plant cells to maintain precise homeostatic regulation of intracellular auxin level. There are few studies which investigated the absorption and metabolism of exogenous auxins by green algae. Experiments performed on *Caulerpa paspaloides* revealed the presence of dioxindole-3-acetic acid, which in several plant species is known as the IAA catabolite produced via oxidation pathways (Jacobs 1993). Another data obtained from *Nitella* showed that exogenous IAA was predominantly converted into degradation products and other inactive metabolites (Cooke et al. 2002). Additionally Dibb-Fuller and Morris (1992) showed that *Chlorella pyrenoidosa* cell can uptake IAA from external solution and then metabolise it into inactive molecule. This IAA uptake was strongly dependent on pH of the medium and increased sevenfold when pH decreased from 6.0 to 4.5. Based on available data it can be assumed that *C. vulgaris* probably must primarily regulate free auxin levels via balance between the biosynthesis of new auxin molecules and degradation of the existing or introduced auxin analogues e.g. IM. This interpretation is supported by the observation that IAA is synthesised endogenously by *Chlorella pyrenoidosa* and *Scenedesmus armatus* and released to the medium influencing the growth and metabolism of other algal cells in the culture (Mazur et al. 2001).

In addition to the above presented facts, Grossmann (2000) proposed that both natural and synthetic auxins added upon larger amounts induced the phytohormone ethylene, which in turn triggered the biosynthesis of another plant hormone abscisic acid (ABA). This model proposed that auxins at high concentrations increase the activity of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, the key regulatory enzyme in ethylene biosynthesis. For example corn coleoptiles treated with NAA and 2,4-D produced a significant amount of ethylene finally leading to suppression of elongation and photosynthetic pigments degradation (Sunohara and Matsumoto 1997).

The analysis of dose–effect relationships indicated that IM at the concentration range of  $10^{-8}$  to  $10^{-4}$  M had a stimulating effect on algal growth expressed as cell number and dry mass as well as changes in chemical components' levels. Particularly,  $10^{-7}$  M

IM possesses the most promotive influence on cell division, algal viability and anabolism of *C. vulgaris*.

The most characteristic responses elicited by natural and synthetic auxins comprise the stimulation of cell division in higher plants and algae (Stirk and Van Staden 1997). This paper provides evidence that IM, synthetic analogue of IAA, might also have been engaged in the mitosis induction of unicellular green alga. Synchronous culture of *C. vulgaris* showed a significant increase in cell number and dry weight in response to optimal dose of IM ( $10^{-7}$  M) on the 5th day of cultivation. Our results confirm the data obtained in experiments performed on *Caulerpa prolifera* (Chlorophyta) when natural auxin IAA at  $1 \mu\text{mol dm}^{-3}$  affected the optimal growth stimulation (Jacobs 1993). Moreover, adding IAA to microalgae *Scenedesmus obliquus* and *Scenedesmus armatus* culture resulted in the stimulation of cell division, growth and formation of four-celled rather than two-celled colonies (Mazur et al. 2001).

It is known well that auxin-induced growth involves specific changes in gene expression. An exposure of an algal cell to exogenous IM induces the nucleic acid accumulation. It could have been speculated that an increase in DNA level up to 48% per cell undergoes during asynchronous initiation of DNA synthesis and subsequent nuclear division. Probably *C. vulgaris* in response to IM can form cells with three and six autospores in addition to the expected two and four leading to an increase in cell number in the culture (Mandalam and Palsson 1997). Higher total RNA accumulation in *C. vulgaris* cells in response to IM is also consistent with observations that auxin application to whole plants, excised organs or cell cultures leads to a rapid increase in levels of numerous mRNAs (Raghavan et al. 2006). In vitro transcription studies with isolated nuclei showed that accumulation of those mRNA species was due to enhanced transcription rates and 2,4-D-dependent stimulation of RNA polymerase II activity (Memes et al. 1992).

An increase in the level of nucleic acids often contributes significantly to the support of protein synthesis, thus *C. vulgaris* cells treated with  $10^{-7}$  M IM contain and excrete to the environment about 20–43% more water soluble proteins. The obtained results are in agreement with the previous data indicating that natural (IAA) and synthetic auxins such as ILA (indole-3-lactic acid) and IBA (indole-3-butyric acid)

binding site: a planar aromatic ring-binding platform, a carboxylic acid-binding site, and a hydrophobic transition region that separates the two binding sites. Thanks to these properties IM could have been probably regarded in the future as plant growth promoting substance intensively involved in the metabolism and development of green microalgae, one of the important species in freshwater ecosystems responsible for primary production. However, further studies are necessary in order to validate this hypothesis.

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