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Insights into microalgae mediated biodegradation of diazinon by *Chlorella vulgaris*: Microalgal tolerance to xenobiotic pollutants and metabolism

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ABSTRACT

Diazinon is one of the most widely used organophosphorus insecticides for agricultural activities, and it is highly toxic to mammals and other non-target organisms. The present study demonstrated the effective removal of diazinon from the aqueous phase by a freshwater, green microalga, *Chlorella vulgaris*. Among the four screened species (*Scenedesmus obliquus*, *Chlamydomonas mexicana*, *Chlorella vulgaris* and *Chlamydomonas pitschmannii*), *C. vulgaris* showed the highest removal capacity (94%) of diazinon at 20 mg L^{-1}. The growth of *C. vulgaris* was significantly affected above 40 mg L^{-1} of diazinon, showing >30% growth inhibition after 12 days of cultivation. Significant enhancement of the microalgal growth in the exponential growth phase suggested a less/non-toxic nature of the diazinon by-products. Biochemical properties, including carotenoid, chlorophyll *a*nd antioxidant enzymes of *C. vulgaris* were influenced by diazinon at relatively high concentrations. The degradation rate constant (k) and the half-life ($T_{1/2}$) of diazinon (0.5–100 mg L^{-1}) ranged between 0.2304–0.049 d^{-1} and 3.01–14.06 d, respectively. Gas chromatography mass spectroscopic (GC–MS) study suggested the formation of a less toxic by-product, 2-isopropyl-6-methyl-4-pyrimidinol (IMP) as a result of microalgal metabolism of diazinon. This study demonstrated that *C. vulgaris* is highly tolerant of diazinon, which could be voluntarily involved in the removal of traces of diazinon from contaminated wastewater and has potential application in the removal of such artificial toxins using algae.

1. Introduction

Approximately one-fifth of the world's population does not have access to safe water, and two-fifths suffer the consequences of unacceptable sanitary conditions due to contamination of water resources with various synthetic and geogenic compounds, which are being leached from agricultural, industrial and domestic activities [1]. It is therefore, obvious that chemical pollution of freshwater reserves is one of the crucial ecological concerns facing humanity in almost all parts of the world. The agricultural sector is one of the most diffuse sources of aquatic pollution, with >140 million tons of fertilizers and several million tons of pesticides, insecticides and herbicides applied each year [2]. The chemical stability and the large application of these compounds have caused a serious level of contamination of natural waters [3,4].

Diazinon [O,O-diethyl O-(2-isopropyl-6-methylpyrimidin-4-yl) thiophosphate] is an insecticide belonging to the organophosphorus (OP) chemical family with widespread agricultural and non-agricultural uses. The annual use of diazinon in the United States alone is recorded as high as ~6 million kilograms [5]. Large amounts of diazinon residues and its metabolites have been frequently detected in various aquatic systems, including effluents from sewage- treatment plants and urban waterways all over the world [6–8]. Diazinon has been classified by the World Health Organization (WHO) as a moderately hazardous Class II chemical due to its potential toxicity [9]. It inhibits the acetylcholinesterase enzyme and causes potentially harmful health effects, which include mutagenicity, cytotoxicity, neurodevelopmental, genetic malformations, immunological disorders and endocrine-disruption in mammals [4,5]. The ecotoxicological effects of diazinon on microalgae have been studied earlier. The photosynthetic apparatus in microalgal cells can be greatly disrupted due to the presence of diazinon [30]. The harmful effects of diazinon on the production of ATP in several microalgal species, including *C. ellipsoidea*, *E. elastica* and *Chlamydomonas* sp., are evident [31]. Considering the toxicity and less volatile nature of OPs, such as diazinon, their removal from drinking water using suitable treatment processes is a high-priority issue for safe drinking water supplies [12,13].

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A number of conventional physicochemical methods, including adsorption, nanofiltration, ozonation, ultrasonic irradiation, photocatalysis and recently, advanced oxidation processes, have been developed for the purification of water containing diazinon [7–9,14–16]. Although these methods are quite effective and offer several advantages, the cost associated with these treatments is still questionable. Moreover, the byproducts formed after degradation of diazinon (especially diazoxon) are more toxic than its parent compound [5,9,16]. On the other hand, biotechnological approaches are the most suitable alternative methods for tackling this aquatic pollution in an ecofriendly manner [13,17,18]. The ubiquity of algae and cyanobacteria in different habitats, and evidence of their existence in various stromatolites of the Archaean and the Mesozoic era, give credence to the theory that these are one of the ancient organisms to appear on the planet Earth [19]. However, the inherent potential of these species for bioremediation has been neglected by environmentalists [19–21]. In particular, mixotrophic microalgae with the dual roles of renewable energy captured by CO_2 fixation through photosynthetic process and biodegradative potential of emerging organic compounds (EOCs) can be unique bioremedial agents. Microalgae can be successfully utilized for remediation of various pollutants because of their versatile metabolic diversity and capability to switch in response to the environment [22–26]. Nonetheless, in-depth mechanistic investigations and scientific evidence on microalgae application in degradation of xenobiotic organic pollutants lag far behind than that of bacteria/fungi.

Considering the aforementioned facts, the current study was aimed to evaluate the influence of an organophosphorus insecticide, diazinon, on the freshwater green microalgae *Chlorella vulgaris*. The changes in the growth rate, biochemical characteristics and antioxidant enzyme activity of microalga were monitored, and the potential of *C. vulgaris* for biodegradation of diazinon was also evaluated. To date, relevant information on the degradation mechanism of microalgae has been scarce in the literature. Therefore, a mechanism of biodegradation of diazinon was proposed by identifying the metabolites obtained after the biodegradation of diazinon using gas chromatography mass spectroscopy (GC–MS).

2. Materials and methods

2.1. Chemicals

Diazinon was obtained from Sigma-Aldrich (St. Louis, MO, USA) and all the metal salts used for this study were purchased from Duksan (Seoul, South Korea). HPLC grade-methanol, water and acetonitrile were purchased from Thermo Fisher Scientific (USA). All the chemicals used were of the analytical grade and highest purity available.

2.2. Algal strains, culture conditions and inoculum preparation

The microalgal species *C. mexicana* GU732420, *C. vulgaris* FR751187, *C. pitschmannii* GU732416 and *S. obliquus* GU732426 were investigated in this study. Each microalga strain was individually inoculated in a 250-mL Erlenmeyer flask containing 100 mL of Bold's basal medium (BBM) in sterile conditions, and then cultivated in a shaker incubator at 150 rpm and 27 °C for two weeks. Microalgae were cultivated under continuous illumination using a fluorescent light having a photon flux of 45–50 μmol photon m^{-2} s^{-1}. The microalgal suspensions were adjusted to an absorbance of 1.0 at an optical density (OD) of 680 nm, as measured by a DR/3900 visible spectrophotometer (Hach, USA) to use as an inoculum for further experiments [26].

2.3. Microalgal screening for diazinon removal

Microalgal species were screened according to their removal efficiencies of diazinon. Erlenmeyer flasks containing 100 mL of BBM added with 20 mg diazinon L^{-1} were individually inoculated with 1% of the aforementioned stock cell suspension of microalgae ($V_{inoculum}/V_{media}$) (OD-1.0). The flasks were kept at 27 °C and 150 rpm in a shaker incubator under the white fluorescent light illumination (45–50 μmol photon m^{-2} s^{-1}) for 12 days. Two milliliters of the samples were recovered from culture at regular time intervals to determine the removal of diazinon. As *C. vulgaris* showed the highest removal efficiency of diazinon among the other microalgal species, this species was selected for further investigation.

2.4. Measurement of cell growth and biochemical content

The growth of *C. vulgaris* was observed according to the intensity of optical density at 680 nm. A microalgal suspension (10 mL) was filtered through Whatman filter paper (GF-52) and dried overnight at 105 °C. After cooling to room temperature, dry cell weight of microalgae was calculated by measuring the difference between the weight of blank filter papers and filter papers with microalgal cells. A relationship between OD_{680} and dry cell weight (DCW) (g L^{-1}) of *C. vulgaris* was established using the following equation:

$$\text{Dry cell weight}\left(\text{g L}^{-1}\right) = 0.1447 \times \text{OD}_{680} + 0.0093 \left(R^2 = 0.98\right)$$

The specific growth rate was calculated by using the following equation [27]:

$$\mu = \frac{\ln N_1 - \ln N_0}{t_1 - t_0}$$

where N_1 is the dry cell weight at time t_1 and N_0 is the dry cell weight at time t_0 (day 0).

The total chlorophyll content was measured using an earlier reported methodology [28]. Briefly, a 10 mL microalgal culture was centrifuged at 4500 rpm for 10 min at 4 °C using a cooling centrifuge (Union 32R Plus, South Korea) to collect the cell pellet. The pellet was re-suspended in 10 mL of 90% methanol, incubated at 60 °C for 10 min, and then centrifuged again for 10 min at 4500 rpm. The absorbance of the supernatant was measured at 665, 652 and 470 nm using a visible spectrophotometer (DR/3900, Hach, USA). The total chlorophyll and carotenoid content of the extract was calculated using the following formulas:

$$\text{Chlorophyll } a \left(\text{mg L}^{-1}\right) = 16.82A_{665} - 9.28A_{652}$$

$$\text{Chlorophyll } b \left(\text{mg L}^{-1}\right) = 36.92A_{652} - 16.54A_{665}$$

$$\text{C}_{\text{carotenoid}} \left(\text{mg L}^{-1}\right) = \frac{1000A_{470} - 1.91\,C_a - 95.15\,C_b}{225}$$

2.5. Extraction and determination of antioxidant enzymes

Assessment of antioxidant enzymes is necessary to estimate the microalgal cells' tolerance and response to diazinon. For this purpose, 5 mL of microalgal suspension was withdrawn from the culture at a regular time interval and centrifuged at 4500 rpm for 10 min at 4 °C. The biomass pellet was washed with distilled water to remove unnecessary traces of the medium and then centrifuged again. The recovered microalgal cell pellet was resuspended in 0.1 M Tris–HCl (pH 7.4), sonicated for 5 min (Medium 200 W, 10 s of sonication strokes with 10 s of intervals) at 4 °C and centrifuged at 10,000 rpm for 10 min. The cell lysate supernatant collected after the centrifugation was used to determine the activities of superoxide dismutase (SOD) and catalase activity (CAT) using assay kits (Cell Biolabs, San Diego, CA, USA). The amount of enzyme that caused a 50% decrease in the nitroblue