Biodegradation of Pesticides DINOCAP and DNOC by Yeast Suspensions in a Batch System

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Nitroaromatic compounds constitute a major class of widely distributed environmental contaminants. Many of these pollutants, including 4,6-dinitrocresol (DNOC) or Karathane (Dinocap) have been widely used pesticides that persist in some contaminated soils, and have been found in ground-waters, causing health and environmental hazards. Dinitrophenols have multiple biological effects, being used in agriculture as insecticides, fungicides, herbicides and acaricides or in medicine and biology as metabolic inhibitors. The environmentally noxious dinitrophenols and related compounds act as uncoupling agents of oxidative phosphorylation, inhibiting ATP formation within the respiratory chain. An anaerobic consortium of different bacterial species is able to completely degrade dinitro-derivatives. However, we investigate here the bioremediation of dinitrophenol contaminated environments with the yeast Saccharomyces cerevisiae, since yeast it is cheap, easily available and largely investigated. Consequently, we have treated 10^{-3} M to 10^{-2} M solutions of two dinitrophenol pesticides (Dinocap and DNOC) with 5g/L yeast suspensions for 1 week in a batch system. The resulted supernatants were used to treat wheat seeds in germination experiments. They displayed different toxicity patterns according to their structure.

Keywords: bioremediation; yeast; pesticide; dinitrophenols; germination test

Hazardous compounds in the contamination of the food supply from agricultural waste are an increasing concern worldwide. Dinitrophenols (DNP) and their derivatives are known to be toxic to the environment, being potent uncoupling agents [1-6]. Despite their overwhelming toxicity, some dinitrophenols are still used as pesticides with an important role in the chemical protection of plants [7]. These compounds act on respiratory process by blocking the reactions of oxidative phosphorylation, which results in the inhibition of ATP formation from ADP [8-9]. Trace amounts of nitrophenols have been found in spiked tap water, reservoir water and stream water [12]. Some anaerobic microorganisms degrade and use them as nitrogen source [10], whereas many studies refer to *Pseudomonas* sp. and *Alcaligenes* sp. strains [11]. However, some technical problems remain to be solved before use bacteria as a tool for hazardous-waste management. A special case is DNP toxicity to the environment, which is manifested during production, conditioning, transportation, storing and, especially, usage of these products or by manipulating and consumption of treated products [1,13]. DNP pesticides proved also to be noxious to higher plants in germination experiments [6]. DNP likely affects the biostructural level of eukaryotic organisms [14,15]; therefore, some coupled germination experiments could be reliable tools to reveal their toxicity to the environment [16-20]. Thus, several dinitrophenolic compounds such as Karathane, DNOC, 2,4-dinitrophenol have been tested within the germination experiments comparatively to some dinitrophenyl ethers namely dinitroanisol, 2,4-dinitro-1-(octadecyloxy)benzene, 3-(2,4-dinitrophenoxy)propan-1,2-diol [6]. These results demonstrated that germinating seeds are very sensitive toward DNP toxicity.

We proposed here the use of *Saccharomyces cerevisiae* as a DNP biodegradating and bioaccumulating material, due to its easy availability and reduced cost, as shown previously [21-23]. Yeast cells hold the property to shift quickly from respiration to fermentation, being less sensitive to low concentration of dinitrophenols. The biodegradation events of the investigated compounds were followed by UV-Vis and infrared spectroscopy, as well as germination assays using the supernatant containing degraded dinitrophenols.

Experimental part

Materials and methods

Reagents

We used in this study analytical grade reagents, while all solutions were prepared with milliQ grade water with R = 18.2 Ω. DNP were purchased from Sigma-Aldrich (USA), unless otherwise specified. Dinocap (Karathane-Krt), which is a mixture of 2,4-dinitro-6-octylphenyl crotonates and 2,6-dinitro-4-octylphenyl crotonates, octyl here being a mixture of the methylheptyl-1-ethylhexyl-and 1-propylpentyl-isomers, was purchased from a crop protection shop in Iasi. In general, we compared two dinitrophenolic pesticides, DNOC and Krt, with other dinitrophenols prepared both under classical conditions and under microwaves [24-27].

Biological materials

Baker's yeast was purchased weekly from SC Rompak srl Pascani (Romania), and kept in a humidor at 4 °C. *Triticum aestivum* wheat seeds, *Gasparom* variety, have been taken from Suceava Agricultural Research Station.

Instruments.

A Libbra S35 PC UV/VIS spectrophotometer (Biochrom, UK) was used for spectral measurements. For both UV and VIS-domains quartz cuvettes (Helma/Müllheim) with a 1-cm path length were used [28-30]. Mass spectrometric analysis was performed using a Shimadzu GCMS-QP2010

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quadrupole mass spectrometer, which detects samples via electron impact ionization (EI) or chemical ionization using methane (CI). The HANNA PH 211 microprocessor pH meter was used for pH measurements.

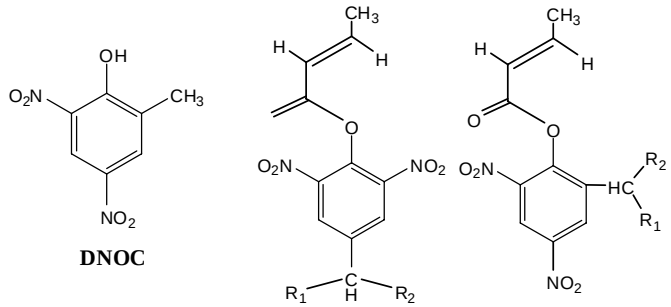
Procedure

Suspensions with (5 g L^{-1}) or without yeast and 50 g L^{-1} glucose, 0.40 g L^{-1} KH_2PO_4 and the corresponding 10^{-3} M and $5 \cdot 10^{-3}$ M investigated compound were introduced, in three replicates, in 300 mL Erlenmeyer flasks. The biodegradation process occurs 7 days on an IKA-KS 4000 ic control orbital stirrer at 25°C and 50 rpm. For investigated compounds were selected: DNOC and Krt (fig. 1). Seven days after the starting of the biodegradative process, yeast suspensions were centrifuged for 10 min at 6000 rpm using a Mikro 22 R centrifuge (Hettich, Germany). The supernatants were used in spectroscopic measurements and in germination experiments. Separately, the content of supernatants were extracted with ethyl ether to separate both remanent dinitrophenols and the resulted compounds in the degradation process. The etheric solutions were evaporated to dryness, then used in thin layer chromatography (Kieselgel 60 F_{254}, Merck) or flash chromatography. The obtained fractions have been characterized by spectroscopy and mass spectrometry.

Dose-response germination experiments included seed germination and analyses of shoot and root elongation on paper [6, 16-20]. Thus, 7-day wheat plantlets were harvested from their seeds, measured (height **H**, expressed as cm) and weighed (**M**, expressed as grams).

Statistics

All findings were validated using the Tukey test [31].



R_1 - methyl, ethyl, propyl Dinocap-4 (**Krt.**) Dinocap-6 (**Krt.**)

Fig. 1. The chemical structure of investigated dinitrophenolic pesticides: DNOC (2-methyl-4,6-dinitrophenol), and Krt(Dinocap), (*RS*)-2,6-dinitro-4-octylphenyl crotonates and (*RS*)-2,4-dinitro-6-octylphenyl crotonates)

Results and discussions

Biodegradation process by Saccharomyces cerevisiae. During the 7-day contact with dinitrophenols, the colour of yeast suspensions changed severely (fig. 2). The absorption maxima and the intensity of each suspension was dependent of the type of pestide used and its concentration. The suspensions treated with DNOC had also different colours, including that with the concentration of 10^{-3} M. The same colour but more intense was developed in the suspension treated with $5 \cdot 10^{-3}$ M DNOC. It is clear that the same biodegradation reactions occurred. Dinocap (Krt) had different biodegradative pathway, since the concentration of 10^{-3} M Krt did not change the colour of yeast suspensions, whereas $5 \cdot 10^{-3}$ M Krt changed the colour of suspensions to yellow. We suspected that at higher Krt concentrations low amounts of pesticide were hydrolyzed and then thoroughly reduced to mono-nitroderivatives, which are not toxic toward wheat seeds. The yellow colour of $5 \cdot 10^{-3}$ M Krt

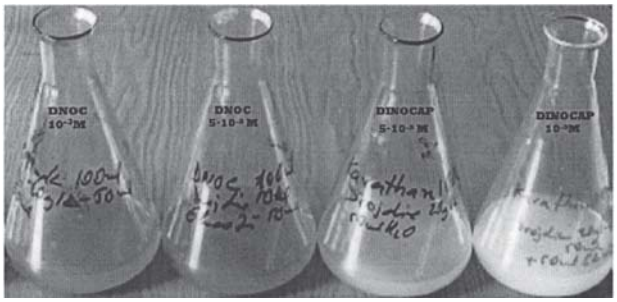


Fig. 2. Batch yeast (*Saccharomyces cerevisiae)* degradation of DNOC and DINOCAP (Krt) within the 7-day experiment. Pesticide concentrations: 10^{-3} M, and $5 \cdot 10^{-3}$M, respectively

suspensions suggest that the reduction was incomplete. Thus, the remaining dinitrophenols resulted during hydrolytic process could be noxious to plants.

Germination tests with supernatants containing degraded DNOC

As low as 10^{-3} M aqueous solutions of DNOC inhibit wheat growth, while DNOC-treated yeast suspensions were completely non-toxic to wheat seeds and seedlings (fig. 3). Yeast suspensions lacking glucose could have a stimulatory effect on wheat development during germination experiments. In contrast, the glucose induced a less stimulatory effect on wheat seedlings. Possibly, glucose depleted in the presence of yeast in suspensions, and some by-products could have a toxic effect on wheat seeds and seedlings.

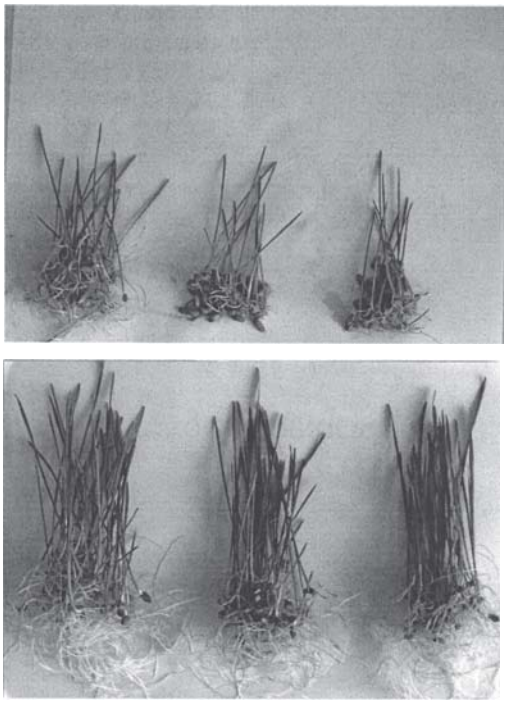


Fig. 3. Effect of DNOC (*left*) and supernatants from DNOC biodegradation *(right)* on wheat seeds during the 7-day germination process

Chemical pathways for the investigated dinitrophenols during the degradation process were followed by measuring the UV-visible changes of supernatants. In our experiments, $5 \cdot 10^{-3}$ M solutions of DNOC displayed four absorbance maxima, at 212 nm (A = 0.648), 268 nm (A = 0.566); 370 nm (A = 0.571), and 411 nm (A = 0.425), respectively (fig. 4). The supernatant from DNOC-treated yeast suspensions containing glucose, displays the following absorbance values: A = 0.343 (212 nm), A = 0.183 (268 nm), A = 0.283 (370 nm), and A = 0.277 (410 nm), respectively.

Moreover, in the absence of glucose, the following values were measured: A = 0.696 (212 nm), A = 0.204 (268 nm), A = 0.137 (370 nm), and A = 0.277 (411 nm), respectively. In brief, the band at 212 nm disappeared due to yeast biodegradation, while the maxima at 268 nm and 410 nm of pure DNOC in solution were shifted to 263 nm and 411 nm, on using yeast. In the presence of glucose the above mentioned maxima shifted toward 272 nm and 431 nm, respectively. In addition, the absorbance at 268 nm decreased from 0.566 to 0.203. The spectra were normalized in order to compare the supernatants.

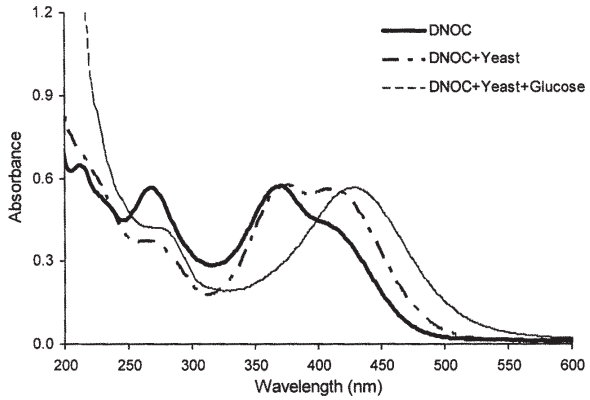


Fig. 4. Degradation of DNOC by yeast suspensions (C_{DNOC} $5 \cdot 10^{-3}$ M). Yeast changed dramatically the composition of supernatants, some non-degraded DNOC may exist alongside with the degradation products and cell components. The normalized spectra before and after degradation, were represented (used)

Effect of supernatants from Dinocap (Krt)

Solutions of Krt 10^{-3} M did not inhibit wheat growth, whereas the supernatant resulted from Krt treated yeast suspensions was toxic to wheat seeds and seedlings (fig. 5). In fact, aqueous suspensions of Krt were no interfering to wheat development during germination, having a similar effect as control treatment (distilled water). The use of yeast suspensions resulted in an increase in the height of plantlets. Toxicity may be attributed to conversion of Krt into its dinitrophenolic derivatives, that could be more toxic than the parental compound.

Dinocap (Krt) found in 10^{-3} M aqueous suspensions displayed two absorption maxima at 375 nm (A = 0.060), and 420 nm (A = 0.056), respectively, in the UV-Vis spectrum (fig. 6). During the treatment with yeast suspensions, the maxima in UV-Vis spectrum of Krt disapeared whether glucose was used or not. In addition, a new absorption band appeared at 260 nm in both cases (in the presence of glucose A = 0.130; without glucose A = 0.079). The newly formed derivatives should further be identified, since they could be cell components released in the supernatant under the action of Krt. We also assumed that some amounts of Krt were bound on the surface of yeast cells. This fact might explain the low absorbencies of supernatants from Krt biodegradation.

Since DNOC has a toxic effect on wheat, the yeast was used to degraded it. The supernatants resulted from DNOC biodegradation process had a slightly stimulatory effect on wheat germination and growth (table 1). For example DNOC treated with yeast induced an increase in the total height of plantlets from 170.5 to 383.9 cm. On treating DNOC with yeast in the presence of glucose, the total height of the plantlets in the lot increased from 117.2 cm to 306.9 cm. The biodegradation of DNOC and the fermentation products had probably opposite effects on wheat development. It was obviously that the resulted supernatant from Krt biodegradation was more noxious than untreated Krt; a drasticaly decrease in the height of plantlets from 344.1 to 132.9 cm was observed. Presumably, Krt was hydrolyzed to active dinitrophenols.

DNOC and pH shift of yeast suspensions.

In this report, we compared the effect of DNOC on the *p*H of yeast suspensions with that of other dinitrophenols, such as 2,4-DNP and 2,5-DNP (fig. 7). In this respect, 40 mL of $5 \cdot 10^{-3}$ M of DNOC (*p*H 5.16), 2,4-DNP (*p*H 5.20), or

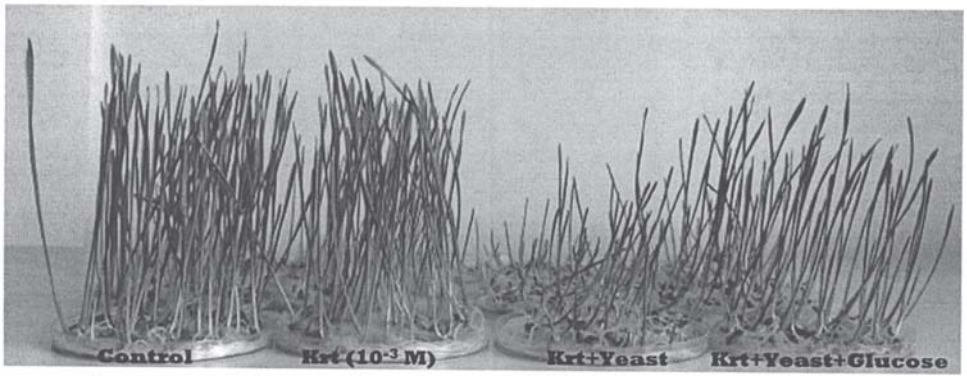


Fig. 5. Effect of supernatants resulted in the Krt biodegradation

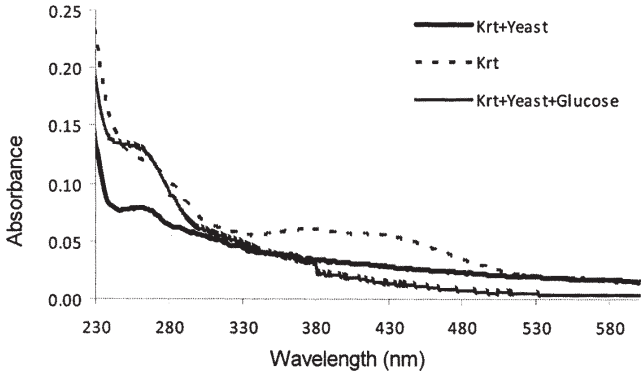


Fig. 6. Yeast-induced biodegradation of Krt followed by UV-Vis spectroscopy

Treatment	Heights of plantlets in 50-seed lots (cm)	Plantlet weight of 50-seed lots (g)	Rootlet weight of 50-seed lots (g)
DNOC 10^{-3}M	117.2±12.45	0.73±0.33	0.025±0.1
DNOC+yeast+glucose	306.9±24.40	1.73±0.14	0.038±0.001
DNOC+yeast	383.9±15.81	2.12±0.14	0.048±0.001
Krt 10^{-3} M	344.1±34.49	1.93±0.19	0.051±0.35
Krt+yeast	132.9±7.50	0.74±0.29	0.025±0.10
Krt+yeast+glucose	238.4±8.40	1.34±0.57	0.028±0.20
Control, H_2O	170.5±11.5	0.95±0.04	0.026±0.20

Table 1
Effect of supernatant derived from the biodegradation of dinitroderivatives on wheat seeds germination

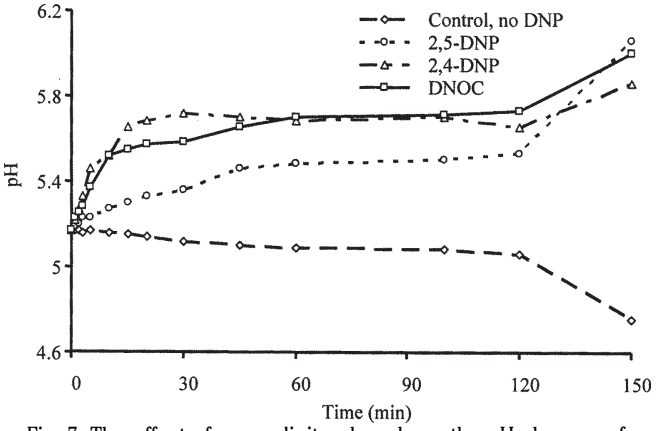


Fig. 7. The effect of some dinitrophenols on the pH changes of yeast suspensions

2,5-DNP (*p*H 5.16) were mixed with 2 mL of 10% yeast suspension, with continuous stirring. The *p*H was monitored with a pH-meter. The *p*H of working solutions and suspensions was adjusted by addition of 1 M solutions of hydrochloric acid or sodium hydroxide to vigorously stirred yeast suspension or DNP solutions. The starting pH of the resulted suspensions was 5.17. DNOC induced a rapid increase in the *p*H value from 5.17 to 5.52 in the first 10 min. The *p*H slightly increases to 5.70 after 1 h and almost stabilized after 3 h (5.73). After one day, *p*H was found to be 6.06. A rather similar effect was observed in the case of 2,4-DNP, with two small remarks: at the end of the first 15 min *p*H was higher (5.68), whereas the *p*H after 24 h was 5.86. A slight increase in Ph was found for 2,5-DNP, probably due to its lower solubility in cell membranes.

Dinitrophenols have been used as body building drugs or analytical tools [32]. However, they may be hazardous materials when are largely used in agriculture and biology. Many hypotheses and theories tried to explain their toxicity based on the properties of dinitrophenyl moiety, which can inhibit the energy transfer from excited molecules in the cell to ATP synthase [5,6]. It is worth to mention that the investigated compounds have a significant absorbance at about 6000 cm^{-1} in the infrared region, corresponding to ΔG of ATP formation. Dinitrophenols also quench the fluorescence of some biological molecules. The toxicity mechanisms of DNP may be related to a decreasing intracellular *p*H [33]. As for the present-date bioremediation approaches, they suffer from a several limitations like the poor capabilities of microbial communities in the field, lesser bioavailability of contaminants on spatial and temporal scales, and absence of bench-mark values for efficacy testing of bioremediation for their widespread application in the field [34]. We consider yeast can be used both *in situ*, in the contaminated area, and in some collecting devices for residual waters from the field. The use of such decontamination procedures will be further presented.

Our results are in best agreement with those obtained by other authors, that measured CO_2 amount produced by yeast fermentation in the presence of various dinitrophenyl derivatives and related compounds [35]. We were also encouraged by the studies of some authors who determined the plant resistance to carbamate herbicides [36], or those who searched for new methods of decontamination of polluted areas [37-42]. Indeed, dinitrophenol pesticides may affect root elongation and shoot growth by concentrations in the range of 10^{-3} – 10^{-2} M, and the germination tests could be effective tools in investigating both pesticide toxicity and the decontamination process.

Conclusions

Yeast suspensions of commercial *Saccharomyces cerevisiae* could be used in the biodegradation of pesticide dinitrophenols and related compounds. Generally, yeast suspensions containing glucose proved to be less effective in decreasing pesticide dinitrophenol toxicity than those without glucose. Thus, a decreased toxicity of the supernatants resulted from DNOC treatment with yeast suspensions was observed in germination experiments. Dinitrophenols pass through yeast cells membrane barrier inducing significant *p*H changes of suspension. In general, yeast slurry can degrade dinitrophenols at concentrations ranging from 10^{-3} M to $5 \cdot 10^{-3}$ M. The effect of resulted supernatants from Krt degradation proved to be more severe than Krt without treatment. Consequently, the biodegradation of dinitrophenolic pesticides should be carefully investigated.

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