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Microcalorimetry as a possible tool for phenanthrene toxicity evaluation to eukaryotic cells

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Abstract

Microcalorimetry and measurement of culture turbidity using a Bioscreen C Analyzer System were applied to study the toxic effect of phenanthrene on *Cunninghamella elegans* IM 1785/21Gp spore germination. The results of *C. elegans* spore incubation in Bioscreen C microbiology reader showed the inhibition of spore germination by 70% (with 25 mg l^{-1} of phenanthrene) and total inhibition of the fungus growth with a higher content of the xenobiotic (50–100 mg l^{-1}). The microcalorimetric technique showed to be useful for the estimation of metabolic activity of *C. elegans* spores in growth medium up to xenobiotic concentrations of 90 mg l^{-1}. These data corresponded with the microscopic observations. The obtained results showed that the microcalorimetry method could be a valuable supplement in the study on the mechanism of PAHs detoxification by fungi.
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Keywords: *Cunninghamella elegans*; Microcalorimetry; Phenanthrene; Fungus; Growth

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are foulants causing great environmental concern because many of them have damaging toxic and carcinogenic features [1]. Filamentous fungi play an important role in degradation and detoxification of PAHs. We have previously documented that the filamentous fungus *Cunninghamella elegans* IM 1785/21Gp has a capability to remove phenanthrene [2].

Recently, *C. elegans* has been widely and profitably used as a microbial model of the mammalian metabolism under the influence of xenobiotics, including PAHs, too [3–5]. It is also convenient to study the toxic effect of phenanthrene on mammalian metabolism, using the fungus as a research model. The study with *C. elegans* can elucidate deleterious effects of the xenobiotic on cell morphology and the growth rate of the model organism.

In order to continuously follow the growth of *C. elegans* spores in the Sabouraud medium and the toxic effect of phenanthrene on the fungal growth, a series of calorimetric measurements was performed using a microcalorimeter. Calorimetry is a direct non-invasive physical technique that allows one to measure the changes in thermodynamic parameters in biological systems. This technique makes it possible, among other things, to record in time at a constant temperature the metabolic processes being a sum of the energetic effects, which take place in biological systems (in our case, the growth of *C. elegans* spores).

2. Experimental

2.1. Chemicals

Phenanthrene was obtained from Sigma (Steinheim, Germany). Dimethylformamide was purchased from Serva (Heidelberg, Germany). All chemicals and solvents were high purity grade reagents.

2.2. Microbial strain and cultural conditions

Cunninghamella elegans IM 1785/21Gp, from the Department of Industrial Microbiology and Biotechnology fungal

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strains collection, was used. The features of this strain had been described in our earlier paper [2]. All tests were carried out at 28 °C using a Sabouraud liquid medium (Difco Laboratories, USA), inoculated with 10-day-old cultures of *C. elegans* IM 1785/21Gp on Sabouraud agar slants.

2.3. Sample preparation

Cunninghamella elegans IM 1785/21Gp was inoculated in the Sabouraud medium, initially containing 5×10^7 spores ml^{-1}, then phenanthrene, dissolved in dimethylformamide (10 mg ml^{-1}) was added into the cell suspension (25–100 mg l^{-1}), as indicated in the text.

2.4. Bioscreen cultivation

The fungal growth was measured by monitoring the culture turbidity (420–580 nm), using a Bioscreen C Analyzer System (Labsystems, Finland). The honeycomb plate wells were filled with 300 μl of *C. elegans* IM 1785/21Gp culture with different supplements of phenanthrene or without phenanthrene (control culture). The incubation temperature was 28 °C. During 48 h of incubation a microscope Axiovert 200 M with confocal scanning module LSM 5 Pascal (Zeiss, Germany) with Nomarski differential interference contrast was used for microscopic observations.

2.5. Microcalorimetry

The calorimetric measurements of the fungal growth were performed with the use of a high sensitivity differential scanning calorimeter Micro DSC III (Setaram, France) based on the Calvet principle. The detection limit was ±0.2 to 2 μW and the baseline stability over 3 days was better than ±2 μW. The measurements were carried out in stainless "closed, batch" vessels with a volume of 1000 μl. The sample and reference vessels were sterilized. The sample ampoule was filled with 300 μl of *C. elegans* IM 1785/21Gp culture with appropriate amount of phenanthrene or without phenanthrene. The reference ampoule was filled with 300 μl of Sabouraud medium. Power–time curves for all measurements were performed at a temperature of 28 °C.

3. Results and discussion

In a previous work [2] we documented that the filamentous fungus *C. elegans* IM 1785/21Gp had a capability to eliminate phenanthrene with a high efficiency (99.4%) at a xenobiotic substrate concentration of 250 mg l^{-1} in Sabouraud medium. The phenanthrene removal was associated with fungal growth. However, an inhibitory effect of phenanthrene on *C. elegans* growth, especially at the stage of spore germination, was also noticed and consequently 24-h-old mycelium was applied as inoculum for PAH containing media.

In order to carry out detailed examination of spores germination in the presence of phenanthrene, a Bioscreen C Analyzer System was used in the present work. This system monitors microorganisms' growth as culture turbidity and is widely used for toxicity measurement and screening of inhibitory or stimulatory agents [6–9]. The results of *C. elegans* spore incubation in the Bioscreen C microbiology reader are presented in Fig. 1.

The introduction of phenanthrene into the growth medium at a concentration of 25 mg l^{-1} reduced the spore germination by 70% (in relation to the control culture). The higher contents of the xenobiotic (50–100 mg l^{-1}) blocked the increase in OD values of the cultures, i.e. the total growth inhibition was supposed. However, microscopic inspection of phenanthrene supplemented cultures elucidated a germination activity of the spores (Fig. 2).

Spores incubated in the growth medium with 25 mg l^{-1} of phenanthrene formed short germ tubes with a bulb in the apical part of the germ (Fig. 2B). No long branched filaments, observed in the control culture without the

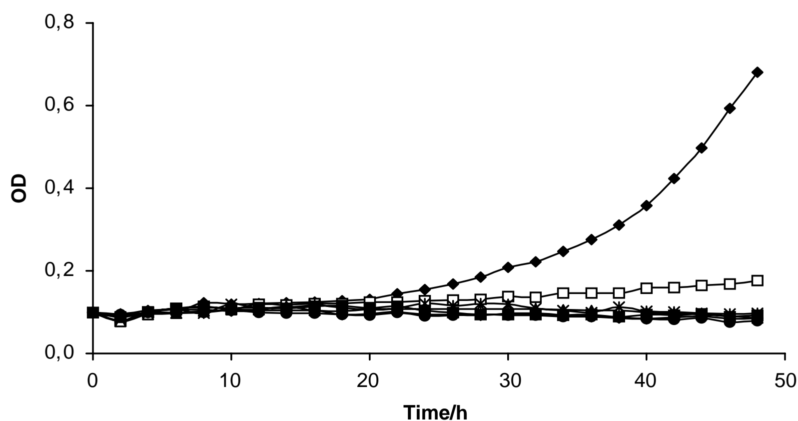

Fig. 1. Courses of *C. elegans* growth in Sabouraud medium without phenanthrene (◆) and with: 25 mg l^{-1} (□), 50 mg l^{-1} (△), 60 mg l^{-1}(■) 70 mg l^{-1} (*), 90 mg l^{-1} (○), 100 mg l^{-1} (●) of phenanthrene.

xenobiotic (Fig. 2A), were found in PAH presence (Fig. 2B). At 50 mg l^{-1} concentration, the xenobiotic stimulated the formation of aberrant swollen spores with very short irregular germ tubes (Fig. 2C). Higher amounts of PAH (60–90 mg l^{-1}) (Fig. 2D–F) blocked germ tube formation but vacuoles and other organelles were observed in contrast to spores incubated with 100 mg l^{-1} of phenanthrene (Fig. 2G) or spores used for inoculation of growth medium (0 h of growth) (Fig. 2, SPI). This observation proves that spores of *C. elegans* are metabolically active up to the xenobiotic concentration of 90 mg l^{-1} in the growth medium.

In order to confirm this supposition, heat production was estimated with a microcalorimetric method in *C. elegans* cultures incubated in the presence of phenanthrene. Calorimetry is one of the scarce physical techniques allowing the measurement of changes of thermodynamic parameters. Calorimetric measurements performed using a high sensitivity calorimeter allow us to register very small changes of temperature in the sample vessels. In our investigations the release of heat was caused by the metabolic process accompanying the growth of *C. elegans* depending on the phenanthrene concentration in the medium. The heat flow changes in time caused by the energetic processes proceeding during the *C. elegans* spore growth without and with the addition of phenanthrene (100 mg l^{-1}) are shown in Figs. 3 and 4, respectively.

The results presented in Fig. 5 revealed an energy flow in the studied fungal samples up to a xenobiotic content of 90 mg l^{-1}, which proved metabolic activity of the incubated spores. The total heat production by spores germinating in growth medium with a phenanthrene amount above 60 mg l^{-1} was significantly lower than that in samples with 25 and 50 mg l^{-1} of xenobiotic or without PAH.

The calculated values of enthalpy change (ΔH) of the metabolic processes taking place during the *C. elegans* spore growth in the Sabouraud medium with or without phenanthrene are given in Fig. 6. As can be seen, even a low

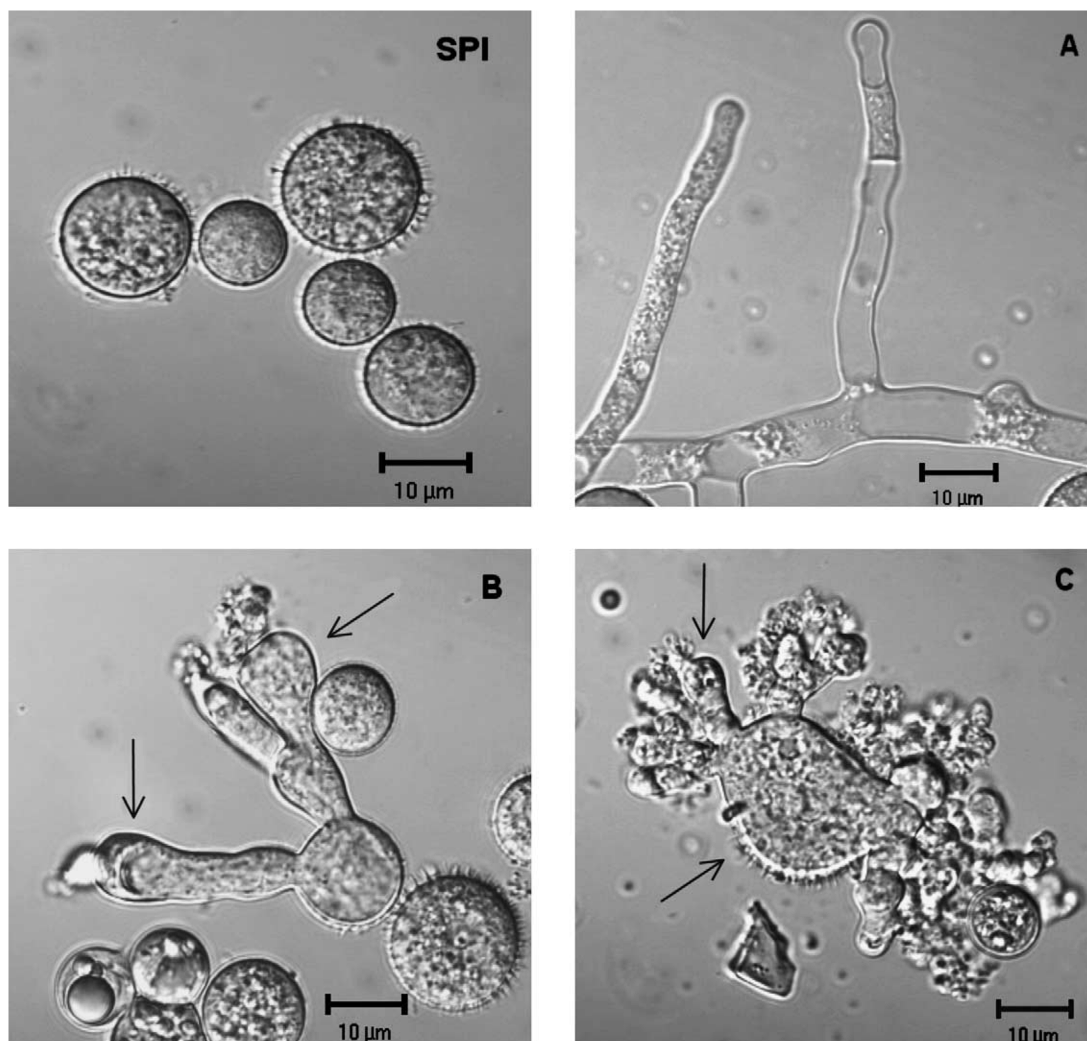

Fig. 2. *C. elegans* photomicrographs. SPI: spores used for Sabouraud medium inoculation (0 h of growth); (A–G) germinating spores after 24 h of incubation in Sabouraud medium without phenanthrene (A) and with 25 mg l^{-1} (B), 50 mg l^{-1} (C), 60 mg l^{-1} (D), 70 mg l^{-1} (E), 90 mg l^{-1} (F), and 100 mg l^{-1} (G) of phenanthrene. Note presence of aberrantly swollen forms (→) and vacuoles (⇒).

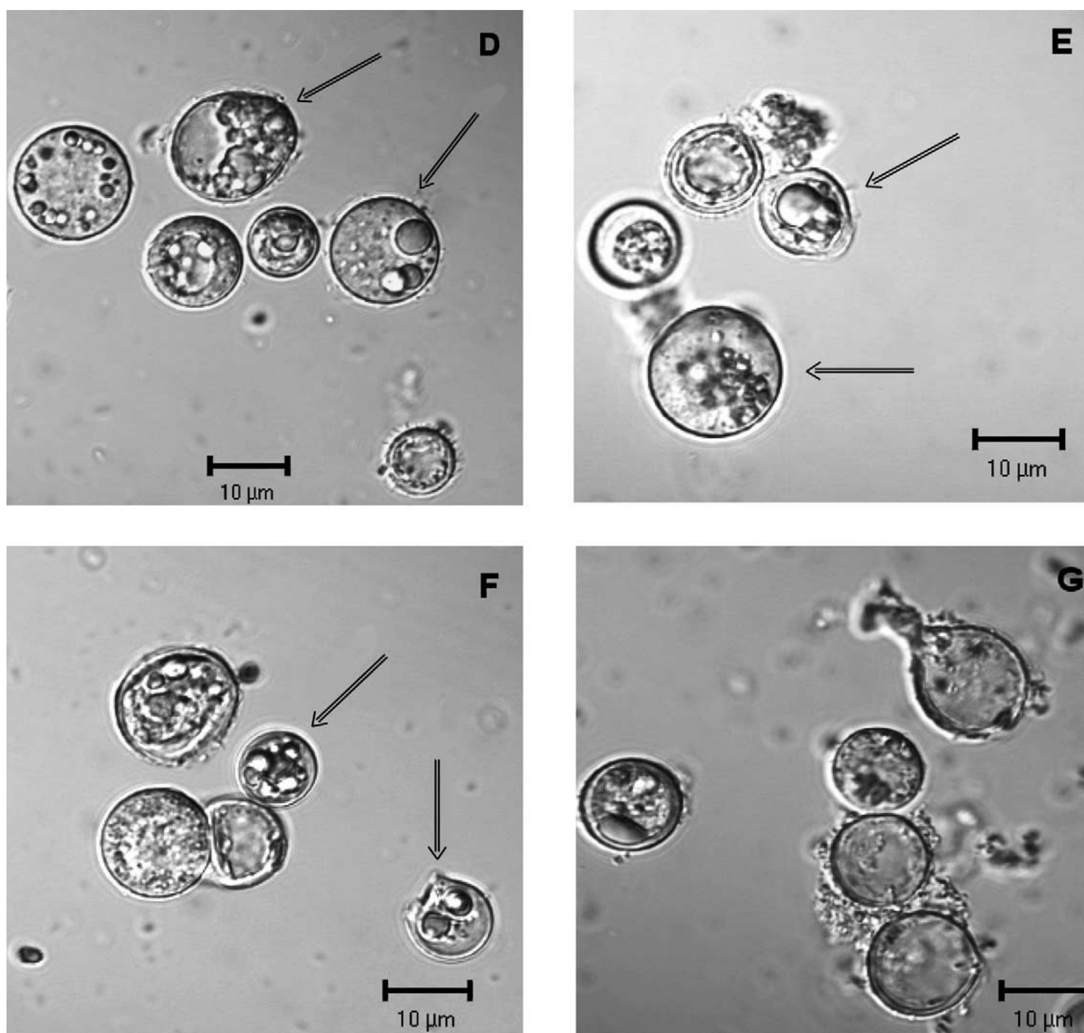

Fig. 2. (*Continued*).

concentration of phenanthrene initiates a drop in the fungal activity and when the phenanthrene concentrations exceeds 60 mg l^{-1}, the fungal growth is almost completely blocked. The control experiment carried out in the absence of external C-source showed that the value of enthalpy change is 0.35 mW. This data allow supposing that the enthalpy change observed at 100 mg l^{-1} of phenanthrene (Fig. 6) is the waste energy from an external C-source degradation.

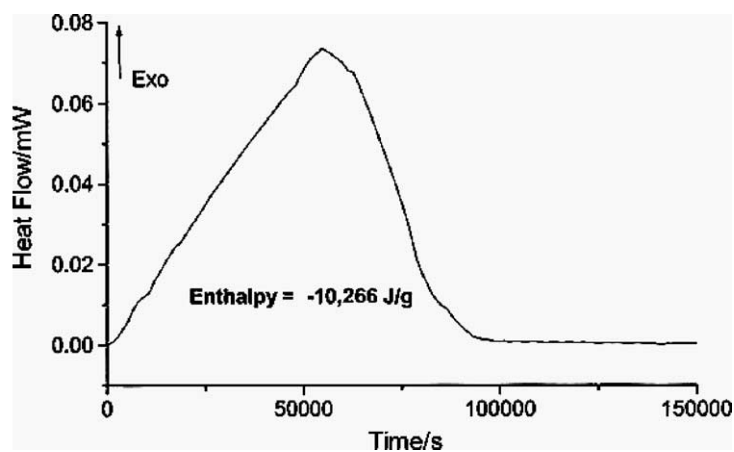

Fig. 3. Heat production by *C. elegans* spores incubated in growth medium without phenanthrene.

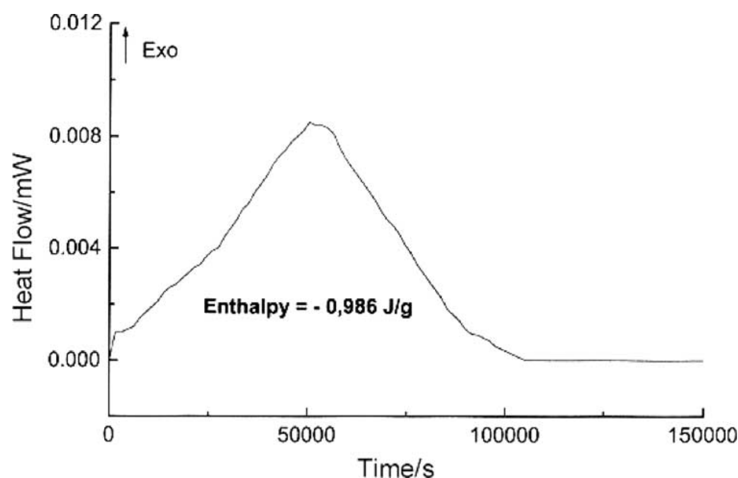

Fig. 4. Heat production by *C. elegans* spores incubated in growth medium with phenanthrene (100 mg l^{-1}).

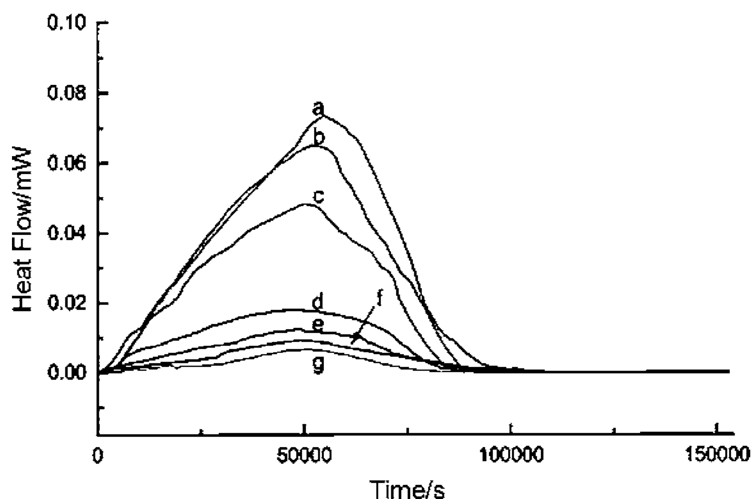

Fig. 5. Heat production by *C. elegans* spores incubated in growth medium without phenanthrene (a) and with 25 mg l^{-1} (b), 50 mg l^{-1} (c), 60 mg l^{-1} (d), 70 mg l^{-1} (e), 90 mg l^{-1} (f), and 100 mg l^{-1} (g) of phenanthrene.

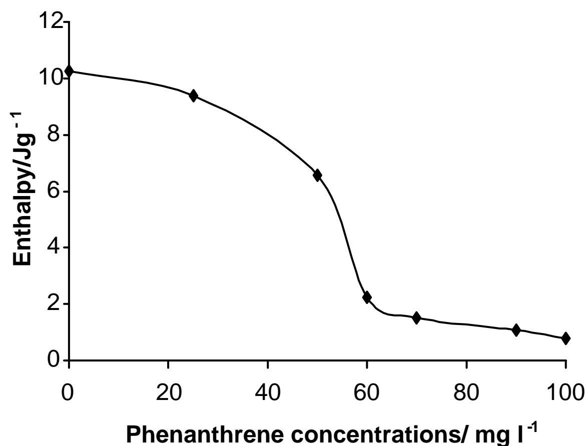

Fig. 6. Relation of total amount of heat production (enthalpy) by *C. elegans* spores vs. phenanthrene content in the growth medium.

These data correspond with the microscopic observation presented above and indicate that although the germ tubes forming is distinctly limited (at xenobiotic concentrations of 25 and 50 mg l^{-1}) the metabolic activity of the aberrantly swollen germs is still high. It remains to be explained whether the irregular *C. elegans* forms also express a significant capability to eliminate phenanthrene.

The obtained results indicate that microcalorimetry as a non-invasive physicochemical method can be a valuable supplement in the study of the mechanism of PAHs detoxification by filamentous fungi. The usefulness of this technique has been also documented in the investigation of microbial degradation of tetraethyl lead in soil [10] and in the study of the toxic action of heavy metals on the filamentous fungus *Rhizopus nigricans* [11,12].

Further microcalorimetric experiments on detoxification processes are being performed in our laboratory.

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