

Inactivation of Possible Fungal Food Contaminants by Photosensitization**

Živilė Lukšienė^{1*}, Dalia Pečiulytė², Sigita Jurkonienė² and Romualdas Puras¹

¹Institute of Material Science and Applied Research, Saulėtekio 9, LT-10223, Vilnius, Lithuania

²Institute of Botany, Žaliojių Ežerų 49, LT-08406 Vilnius, Lithuania

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Summary

Photosensitization is based on the interaction of two nontoxic, nonmutagenic and non-carcinogenic agents – photosensitizer, accumulated in the microorganism, and visible light. This interaction in the presence of oxygen induces radical-based cytotoxic events. The study has been carried out to define a new tool to improve microbial food safety by photosensitization for inactivation of several fungi, which are harmful for food industry and sometimes resistant to other treatments. The obtained data indicate that several microfungi such as *Alternaria alternata*, *Fusarium avenaceum*, *Acremonium strictum* and *Rhizopus oryzae* might be effectively inactivated by this new technology. Clear correlation was observed between the efficiency of inhibition of germination and the amount of photosensitizer, accumulated by the fungus.

Key words: photosensitization, inactivation of microfungi, food processing and safety

Introduction

Recent trends in global food production, processing, distribution, preparation and saving are inducing a growing demand for food safety research in order to ensure a safer global food supply. Nevertheless, the methods and technologies used for microbial control of food are not always efficient enough, nor entirely human and environmentally friendly. Well-known nonthermal technologies can alter the structure of proteins and polysaccharides, causing changes in the texture, physical appearance and functionality of food. For instance, high-intensity ultrasound can also denature proteins and produce free radicals adversely affecting the flavour of fruit-based or high-fat food (1). In addition, higher doses of ionizing radiation may cause slight colour changes in beef, pork and poultry (2). Natural compounds, such as essential oils, chitosan, nisin or lysozyme, are investigated with the aim to replace chemical preservatives and to

obtain »green label« products. So far, their application is mainly hampered by the changes in the organoleptic properties after their introduction in food (3).

Furthermore, the resilience of bacterial spores and the existence of highly resistant microbial subpopulations also limit the efficacy of the emerging nonthermal technologies (4). The risk for viral contamination of food is present everywhere in the process »from farm to fork« (5).

On this background it is easy to draw a conclusion that presently existing methods for inactivation of harmful, pathogenic and sometimes resistant microorganisms in different fields, including food manufacturing and safety, particularly prepared and semi-prepared foods, are really limited and have certain disadvantages. Inevitably, new approach to inactivation of harmful microbes in cost-effective and environmentally friendly way is still a problem.

*Corresponding author; E-mail: Zivile.Luksiene@mtmi.vu.lt

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According to Dougherty, Raab was first to observe the death of *Paramecium caudatum* after the exposure to light when acridine orange was present (6). It was impossible to understand the mechanism of the death a hundred years ago. Recently it has been accepted worldwide that this phenomenon might be named »photosensitization«. In general, this treatment involves a photoactive organic compound (for instance acridine orange, methylene blue or hematoporphyrin) that usually accumulates in target microorganism and subsequent irradiation with visible light. This combination of two absolutely nontoxic elements, dye and light, in oxygenated environment induces damage and total destruction of microorganisms or the target cell it accumulates in. In 1924 this phenomenon was applied to cure skin cancer (7). It is important to note that really major advances have been made in photosensitized antimicrobial chemotherapy, in particular disinfection of the blood and blood products, treating locally infected wounds or oral candidiasis (8). Moreover, numerous investigators demonstrated possible practical usefulness of photosensitization in the broad field of different sciences: virology, microbiology, immunology or dermatology (9–14).

The intention of this study was to determine the sensitivity of several micromycetes that are harmful in food industry to the photosensitization as novel and promising biophotonic technology.

Materials and Methods

Object

In order to evaluate the sensitivity of microfungi to photosensitization by hematoporphyrin dimethyl ether (HPde) and visible light, we selected micromycetes of several strains, belonging to different morphological types, harmful to food industry, plant substrates, grains of corn, *etc.*

Photosensitizers

The stock solution of HPde (the gift from Prof. G.V. Ponomarev, Russia) was prepared in physiological saline (25 mM) and stored in the dark below 10 °C (15).

Irradiation

The light source used for irradiation of microfungi consisted of tungsten lamp (500 W), optical system for light focusing and optical filter for UV and infrared light elimination (370 nm λ >680 nm). Light intensity at the position of the object was 30 mW/cm², irradiation time was 15 min and the total light dose was 27 J/cm² (15).

Fluorescence measurements

Photosensitizer accumulated in the microfungus was detected by fluorescence microscope (Nikon Eclipse E-400). Micromycetes were prepared as usual for photosensitization, incubated for different time intervals (20 min–18 h) with stock solution of HPde (500 µM) and washed by centrifugation with medium without photosensitizer. Excitation of red porphyrin fluorescence was performed using excitation at $\lambda=330\text{--}380$ nm.

Experimental setup and evaluation of treatment efficiency

Stock cultures of fungi were stored at 4 °C on malt extract agar (MEA, Sigma). Petri dishes containing MEA were inoculated with agar plugs obtained from stock cultures. The fungi were cultivated at 25 °C in the dark to achieve typical growth and sporulation. Conidia (or spores of *Rhizopus oryzae*) were harvested by flooding Petri dishes with 10 mL of distilled water and scraping the surface of colonies with glass rods. The resulting suspension was agitated and filtered through double-layered sterile cheesecloth to remove hyphal fragments; the obtained suspension was decanted, replaced with phosphate buffer solution (PBS), and diluted up to 10⁶ spore/mL. Stock solutions of HPde in PBS or in control solution (to obtain 25–710 µM HPde concentration in spore suspension) were added to spore suspensions and incubated at 25 °C for 20 min. After the exposure to visible light, the solutions were decanted and replaced with PBS (1 mL). One sample was irradiated with visible light in the cell culture dishes, while the other sample was left in the dark. At least 3 separate experiments were conducted for each fungus investigated.

After irradiation, the buffer was removed and replaced with 1 mL of fresh medium. Suspensions were spread onto water agar (WA, Oxford) on the glass slides, placed in Petri dishes on the glass rods and incubated in the dark at 25 °C. Plates were incubated for 24–72 h prior to assaying the number of germinated spores (or conidia) for the detection of their survival. Control dishes were prepared to evaluate the spore germination (in %). Five microscope fields were observed on each glass slide of the control, treated only with HPde, and irradiated with light variants. Germinated and non-germinated conidia were counted in random fields at 40× with a light microscope (Motic microscope B1-setsries biological microscopes). A total of 250–300 conidia were counted.

Assessment of conidia germination

After each exposure session the fungal suspension was pipetted on cover slips and placed conidia-side down on water agar medium in 9-cm Petri dishes. The plates were incubated at 25 °C for 24 h to induce conidial germination; afterwards the conidia germination was assessed (16). Germinated and non-germinated conidia were counted with a light microscope (magnification: 100×). A total of 300–500 conidia were examined on each cover slip, with a higher number of conidia in the case of low germination. The conidia were considered germinated if their length was about 20 µm. The percentage of germination was calculated as follows:

$$\frac{\text{number of germinated conidia}}{\text{total number of conidia}} \times 100$$

Assesment of intracellularly accumulated photosensitizer

Microfungi were collected and incubated for 20 min with photosensitizer in phosphate-buffer solution (PBS) to a constant absorbance. The fluorescence spectra of the suspension were measured with a spectrofluorime-

ter CФP-1 (Moscow, Russia) (17). The sample was excited through the interference filter with $\lambda_{ex}=405$ nm and an epi objective. The fluorescence was registered from the front surface of the sample. The constructional features of the device made it possible to measure the fluorescence of a thin layer (less than 1 mm) of the solution without spectrum distortions due to the effects of the intrinsic filter and light scattering. The fluorescence was excited with the radiation of a mercury lamp through an interference filter with $\lambda_{ex}=405$ nm, and was registered at $\lambda=600\text{--}680$ nm with an emission slit of 10 nm. The measurements were made at room temperature. Suspension of microfungi, treated in the same manner without photosensitizer was taken as control. The standard curves were produced by adding known amounts of the photosensitizer in the solution. Protein concentration was determined by Bradford method.

Results

We choose photosensitizer HPde as representative of dicarboxylic porphyrins because it is effective and much more chemically homogenous in comparison with clinically established agent photofrin (PII), which is a mixture of different porphyrins. Chemical structure of this compound is presented in Fig. 1. We investigated several physicochemical properties of HPde and have found that it is less aggregated compound than PII, more soluble in water and has clear fluorescence maximum at 630 nm (unpublished data). By no means, the most important feature of the photosensitizer is the uptake inside fungus. Thus, we tried to find evidence of the effective accumulation (uptake) of hematoporphyrin dimethyl ether in selected microfungi. Therefore, for better visualization, all investigated microfungi were incubated with high concentration of HPde (500 μ M) for some period (20 min–18 h). So far no significant visual

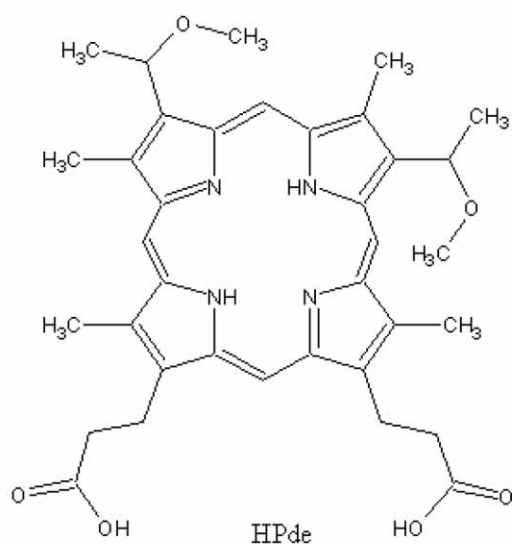


Fig. 1. Chemical structure of hematoporphyrin dimethyl ether (HPde)

difference (distribution of photosensitizer inside the microfungi) was observed using this time interval. In this context, we prefer 20-min incubation time for further investigations. Thus, the fluorescent microphotograph, presented in Fig. 2, clearly indicates that living microfungi are able to accumulate HPde. Moreover, it is understandable that the loci, where HPde is preferentially accumulated, will be destructed after photosensitization. It is worth mentioning that several strains of microfungi show very high fluorescence of HPde located in the conidia (*Rhizopus*, *Aspergillus*, *Fusarium*).

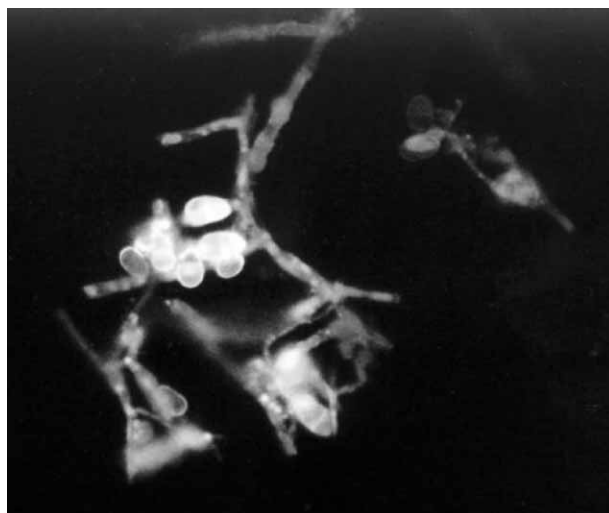


Fig. 2. Fluorescent microphotograph of *Rhizopus oryzae* (400 \times) incubated with HPde. Usually red HPde fluorescence reflects the loci of accumulation (conidia and micelia)

The data presented in Fig. 3 clearly indicate that HPde alone (without light) has no significant inhibition on conidia germination of *Alternaria alternata*. The increase of concentration of this photosensitizer acts on conidia germination, inhibiting it by up to 20 % (510 μ M).

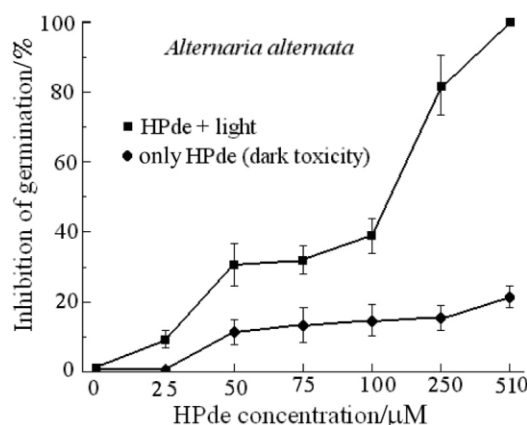


Fig. 3a. Inhibition of *Alternaria alternata* as function of used photosensitizer (HPde) concentration: micromycete was incubated with different concentrations of HPde, afterwards irradiated with visible light (27 J/cm²). Inhibition of conidia germination was the main parameter to evaluate the efficiency of the treatment. The error bars represent the standard deviation of 3–5 repeats

It is interesting to note that following irradiation of this microorganism by visible light increases the inhibition by about 100 %, if compared with the action of HPde alone. Moreover, the data presented in Fig. 3b reveal that *Alternaria alternata* effectively accumulates the photosensitizer. The photosensitizer accumulation degree depends on the concentration of the photosensitizer in the medium: the higher concentration of it is in the medium, the higher the concentration is detected in the fungus.

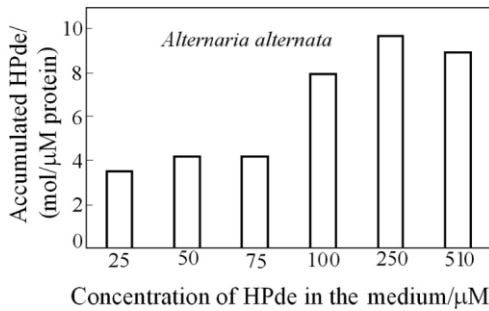


Fig. 3b. Accumulation of photosensitizer by *Alternaria alternata* as function of used HPde concentration in the medium. Accumulation was evaluated as mol/μM protein. Measurements were repeated 3 times

Therefore, other experiments were carried out with *Rhizopus*. Results presented in Fig. 4 suggest that this microorganism is much more resistant to HPde treatment without light if compared with *Alternaria alternata*: it could induce *per se* just 15 % inhibition of spore germination. On the contrary, following irradiation with visible light increased the inhibition up to 100 % when higher concentrations of HPde were used (710 μM). At first sight, both examined objects have an inhibition response to photosensitization that depends on concentration (25–710 μM HPde concentration range). Accumulating capacity of this photosensitizer inside the fungus

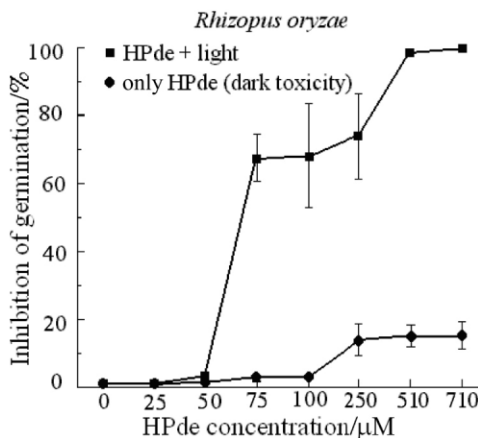


Fig. 4a. Inhibition of *Rhizopus oryzae* as function of used photosensitizer (HPde) concentration: micromycete was incubated with different concentrations of HPde, afterwards irradiated with visible light (27 J/cm²). Inhibition of conidia germination was the main parameter to evaluate the efficiency of the treatment. The error bars represent the standard deviation of 3–5 repeats

as function of used concentration in the medium is presented in Fig. 4b. It is obvious that HPde accumulates more in *Rhizopus oryzae* than in *Alternaria alternata*.

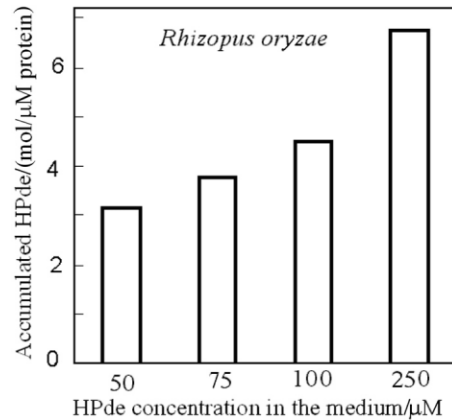


Fig. 4b. Accumulation of photosensitizer by *Rhizopus oryzae* as function of used HPde concentration in the medium. Accumulation was evaluated as mol/μM protein. Measurements were repeated at least 3 times

It was therefore of interest to investigate the sensitivity of *Fusarium avenaceum* to this treatment. Results indicate that under analogous conditions employed in this study, resistance of this microorganism to photosensitization-induced destruction is much higher: even high concentration of HPde (510 μM) inhibited conidia germination of this fungus. As a matter of fact, the dark toxicity of HPde (HPde only) observed in the case of *Fusarium avenaceum* was rather high and reached 40 % (at concentration of 710 μM) (Fig. 5a). The data presented in Figs. 5a and 5b clearly indicate a strong correlation between the accumulated photosensitizer concentration and the inhibition of spore germination.

The last one under investigation was *Acremonium strictum*. As previously described, the HPde action on inhibition of conidia germination was examined first.

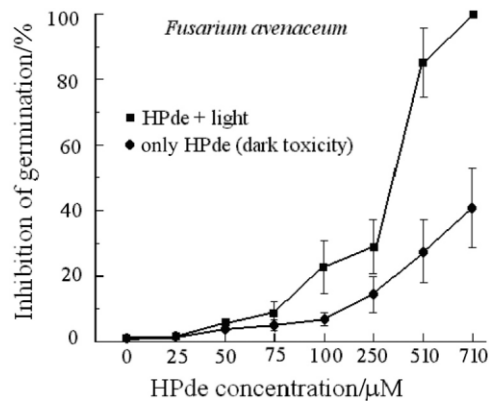


Fig. 5a. Inhibition of *Fusarium avenaceum* as function of used photosensitizer (HPde) concentration: micromycete was incubated with different concentrations of HPde, afterwards irradiated with visible light (27 J/cm²). Inhibition of conidia germination was the main parameter to evaluate the efficiency of the treatment. The error bars represent the standard deviation of 3–5 repeats

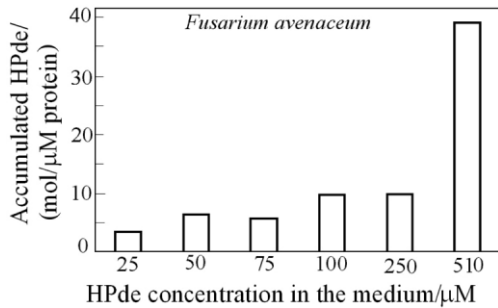


Fig. 5b. Accumulation of photosensitizer by *Fusarium avenaceum* as function of used HPde concentration in the medium. Accumulation was evaluated as mol/μM protein. Measurements were repeated at least 3 times

The data (Fig. 6a) reflect a rather high resistance of *Acremonium strictum* to the action of HPde, only the highest concentrations of this compound (1080 μM) might induce 9 % of germination inhibition. Understandably, a subsequent experiment was performed to evaluate the inhibition induced by photosensitization. As a rule, fairly significant concentration-dependent inhibition of

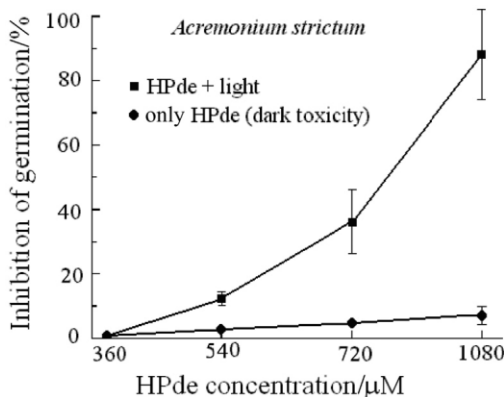


Fig. 6a. Inhibition of *Acremonium strictum* as function of used photosensitizer (HPde) concentration: micromycete was incubated with different concentrations of HPde, afterwards irradiated with visible light (27 J/cm²). Inhibition of conidia germination was the main parameter to evaluate the efficiency of the treatment. The error bars represent the standard deviation of 3–5 repeats

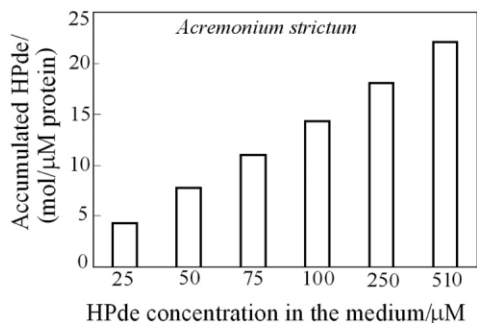


Fig. 6b. Accumulation of photosensitizer by *Acremonium strictum* as function of used HPde concentration in the medium. Accumulation was evaluated as mol/μM protein. Measurements were repeated at least 3 times

conidia germination was observed, and eventually, at concentration of 1080 μM, HPde reached 90 %. Moreover, a correlation between efficiency of inhibition of *Acremonium strictum* and accumulated dye concentration was observed (Fig. 6b).

Discussion

Despite the discovery of antibiotics, thermal and nonthermal technologies for destruction of microorganisms, a struggle against them is still continuing. This is due particularly to wide variety of encountered pathogens and existing disadvantages of methods applied to inactivate them. The development of resistance in a wide range of pathogens is of special importance in this area. In the food processing industry, unwanted occurrence and growth of spoilage and pathogenic microorganisms is a key concern. Thus, new approaches towards this problem seem imperative. In this context, novel, cost-effective, environmentally friendly biophotonic method of photosensitization is available to supplement the armamentarium of existing tools.

So far we have focused on the possibility to inactivate series of harmful and pathogenic microorganisms exploiting photosensitization. In order to evaluate sensitivity of microorganisms to photosensitization by hematoporphyrin dimethyl ether (HPde) and visible light, we selected micromycetes of several strains, which are harmful to food industry, plant substrates, and grains of corn and induce corresponding diseases. For instance, *Rhizopus oryzae* Went & Prinsen Geerl. is distributed in food, indoor (commonly air-borne) and isolated from soil, grain, vegetables, fruits and nuts. *R. oryzae* is the most frequent agent of the human mucormycosis (18). *Alternaria alternata* as well as *Acremonium strictum* are world-wide distributed micromycetes, mostly detected on different substrates: decaying plant, soil, seeds of corn, foodstuffs or air (19). Fungi of *Fusarium* (Link) genus are widely distributed on plants and in soil, as usual components of fungal communities forming on commodities, such as rice, bean, soybean and other crops (20). *Fusarium* fungi are among the most drug-resistant (21).

Our previous data (22,23) indicate that yeast *Saccharomyces cerevisiae* as well as micromycetes *Ulocladium oudemansii*, *Trichotecium roseum* and *Aspergillus flavus* might be inactivated by photosensitization. Moreover, inhibition of spore germination was further observed in *Aureobasidium* sp., *Rhodotorula* sp., *Penicillium stoloniferum*, *Aspergillus fumigatus*, *Aureobasidium pullulans*, *Ulocladium chartarum* (unpublished data). It seems that plethora of harmful micromycetes that destroys food might be inactivated by photosensitization, a method that is completely safe, reproducible, nonmutagenic, noncarcinogenic, environmentally and human friendly. Combination of two absolutely nontoxic constituents of treatment, organic dye and visible light, might really contribute to the inactivation of several fungi and bacteria, the most hazardous enemies in this context. Moreover, from the data obtained it is easy to draw a conclusion that different microorganisms have individual sensitivity to this treatment. For instance, *Aspergillus* can be described as most sensitive to photosensitization by HPde and light:

it was inhibited even at very low HPde doses (23,24). On the contrary, *Fusarium* and *Trichotecium* exhibited certain resistance to this treatment, showing just 30 % of inhibition even when 75 µM HPde concentration was used (23). Nevertheless, further increase of HPde concentration and following irradiation drastically inhibited all investigated fungi up to 80–100 %.

It is accepted worldwide that the amount of accumulated photosensitizer plays a key role in the efficiency of treatment. According to our data, all selected fungi accumulate HPde in significant amounts, up to 30 mol of HPde/µM protein. By no means, the accumulated amount of photosensitizer strictly depends on the dye concentration used in the medium. In all investigated cases clear correlation between the accumulated amount of photosensitizer and its inhibiting activity was observed.

It has been shown convincingly that photosensitization by HPde and light might totally inhibit conidia germination of all investigated fungi. The question arises, what might be the mechanism of this inhibition? It is more or less accepted that the loci, where photosensitizer is preferentially accumulated, are destroyed after photosensitization (6,7,9). The point is that reactive $^1\text{O}_2$, generated during physicochemical steps of photosensitization, has limited migration, and the sites of initial damage after photosensitization are closely related to the localization of the sensitizer (9). Consequently, if a fungus accumulates photosensitizer, it is a sort of guarantee that the object will be sensitive to this treatment. So far, promising and effective methodology for the inhibition of fungal conidia germination and, perhaps, total destruction and elimination of these potentially pathogenic microorganisms seems possible.

Conclusions

In conclusion, the presented data support the idea that photosensitization is an effective tool for inactivation of several harmful and pathogenic microfungi of different strains in nonthermal way. The efficiency of photosensitization has a clear correlation with the accumulated amount of photosensitizer. Higher accumulated concentrations guarantee more efficient destruction of fungus. Thus, the proposed methodology might be used as decontamination tool for different raw materials, foodstuff or other various surfaces in cost-effective, environmentally and human friendly way. Whilst it is not suggested that photosensitization will solve all problems of antimicrobial issues, improvements may be obtained using this new approach in special cases or combining photosensitization with accepted thermal or nonthermal methods for microbial control.

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Inaktivacija mogućih fungalnih zagađivača hrane primjenom fotoosjetljivog spoja

Sažetak

Osjetljivost na svjetlo zasniva se na međusobnom djelovanju dvaju netoksičnih, nemutagenih i nekancerogenih agensa – fotoosjetljivog spoja koji se nakuplja u mikroorganizmima i danjeg svjetla. Njihova interakcija u prisutnosti kisika inducira citotoksične procese zasnovane na reakciji s radikalima. U radu je opisana primjena fotoosjetljivog spoja za inaktivaciju nekih mikroorganizama štetnih u proizvodnji hrane, a često i otpornih na druge postupke. Na osnovi dobivenih rezultata vidi se da se ovom novom tehnologijom mogu djelotvorno inaktivirati mikrofungi: *Alternaria alternata*, *Fusarium avenaceum*, *Acremonium strictum* i *Rhizopus oryzae*. Opažena je jasna korelacija između uspješnosti inhibicije i količine fotoosjetljivog spoja nakupljenog u fungusu.