

ORIGINAL ARTICLE

Inactivation of food pathogen *Bacillus cereus* by photosensitization *in vitro* and on the surface of packaging material

Z. Luksiene, I. Buchovec and E. Paskeviciute

Institute of Applied Research, Vilnius University, Vilnius, Lithuania

Keywords

5-aminolevulinic acid, *Bacillus cereus*, decontamination technology, inactivation, photosensitization.

Correspondence

Zivile Luksiene, Institute of Applied Research, Vilnius University, Sauletekio 10, Lt-10223 Vilnius, Lithuania.
E-mail: zivile.luksiene@mtmi.vu.lt

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Abstract

Aims: The study was focused on the possibility to inactivate food pathogen *Bacillus cereus* by 5-aminolevulinic acid (ALA) – based photosensitization *in vitro* and after adhesion on the surface of packaging material.

Methods and Results: *Bacillus cereus* was incubated with ALA (3–7.5 mmol l⁻¹) for 5–60 min in different environment (PBS, packaging material and wheat grains) and afterwards illuminated with visible light. The light source used for illumination emitted light at $\lambda = 400$ nm with energy density at the position of the cells, 20 mW cm⁻². The illumination time varied from 0 to 20 min, and subsequently a total energy dose was between 0 and 24 J cm⁻². The obtained results indicate that *B. cereus* after the incubation with 3–7.5 mmol l⁻¹ ALA produces suitable amounts of endogenous photosensitizers. Following illumination, micro-organism inactivated even by 6.3 log. The inactivation of *B. cereus* after adhesion on the surface of food packaging by photosensitization reached 4 log. It is important to note that spores of *B. cereus* were susceptible to this treatment as well; 3.7-log inactivation *in vitro* and 2.7-log inactivation on the surface of packaging material were achieved at certain experimental conditions.

Conclusions: Vegetative cells and spores of Gram-positive food pathogen *B. cereus* were effectively inactivated by ALA-based photosensitization *in vitro*. Moreover, the significant inactivation of *B. cereus* adhered on the surface of packaging material was observed. It was shown that photosensitization-based inactivation of *B. cereus* depended on the total light dose (illumination time) as well as on the amount of endogenous porphyrins (initial ALA concentration, time of incubation with ALA).

Significance and Impact of the Study: Our previous data, as well as the one obtained in this study, support the idea that photosensitization with its high selectivity, antimicrobial efficiency and nonthermal nature could serve in the future for the development of completely safe, nonthermal surface decontamination and food preservation techniques.

Introduction

Microbiological food safety is an increasing worldwide problem. An estimated 76 million cases of food-borne disease outbreaks occur annually in the United States, costing 6.5–34.9 billion dollars in medical care and lost productivity (Mead *et al.* 1999).

Clostridium botulinum, *Bacillus cereus* and *Listeria monocytogenes* are the main Gram-positive pathogens

causing food-borne diseases (Altekruse *et al.* 1997). Even spore-forming *B. cereus* has been identified as the cause of 27 000 cases of food-borne illnesses in the United States (Mead *et al.* 1999). *Bacillus cereus* is naturally found in soil environments, because it can contaminate wide range of foods: cereals, fresh vegetables, berries and fruits. Additionally, this pathogen was found in ready-to-eat foods and sauces (Rosenquist *et al.* 2005).

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Heat-stable toxins generated by Gram-positive *Staphylococcus aureus*, *Clostridia perfringens* and *B. cereus* are the common causes of sporadic point-source outbreaks of gastroenteritis (Gill and Hamer 2001).

Several types of antibacterial treatments, traditionally and naturally occurring food antimicrobials are used in food manufacturing. Most of the emerging preservation techniques are based on nonthermal decontamination of food and include ionizing radiation, high hydrostatic pressure, high-intensity pulsed light and natural antimicrobials. Most of them act by slowing down or inhibiting microbial growth, and none can eliminate pathogens totally from the food matrix (Elmnasser et al. 2007; Gomez-Lopez et al. 2007). Taking this into consideration, novel, effective, nonthermal food decontamination technique without any effects on nutritional and organoleptic characteristics of food matrix is of great demand. May be, one of them could be photosensitization.

Since the beginning of the 20th century, when photosensitization-based destruction of highly proliferating living systems was discovered, many attempts have been made in order to understand the mechanism of action of this phenomenon (Hamblin and Hasan 2004; Jori 2006). Recently, it has been accepted that photosensitization (photodynamic therapy, when treating infectious diseases or cancer) is based on the interaction of three factors: photoactive compound (photosensitizer), light and oxygen. As photosensitizers can easily accumulate inside the cells (cancer cells, inflammatory cells or bacteria) with high metabolic activity, after their illumination with visible light, photophysical and photochemical processes inside the cell can induce cell death (Collaud et al. 2004). In detail, a photosensitizer after absorption of light in picoseconds transfer excitation energy from singlet state (S1) to lower excited triplet state T1 with a longer lifetime (milliseconds). Relaxation of photosensitizer from the T1 state results in the induction of two types of photo-oxidative reactions. Type I pathway involves electron or hydrogen atom transfer, producing radical forms of the photosensitizer or the substrate. Type II mechanism is mediated by an energy transfer process to ground state oxygen ($3O_2$). It is worth nothing that the destruction of a cell in this case is strictly localized because of a very short half-life of 102 ns and consequently short diffusion distance of 20 nm (Moan 1990). As a consequence, a plethora of cytotoxic reactions is triggered in the cells. Mostly, cell injuries induced by photosensitization include disruption of cell membrane, inactivation of different enzymes and damage of DNA (Demidova and Hamblin 2005; Luksiene 2005; Jori 2006).

Various studies have demonstrated that some pathogenic micro-organisms can produce endogenous photosensitizers from exogenously applied precursor

5-aminolevulinic acid (ALA; Fig. 1) (Luksiene 2005; Jori 2006; Fotinos et al. 2008). It is clear from Fig.1 that ALA is an endogenous component in the heme biosynthesis pathway, and according to literature ALA is ubiquitous in nearly all cells (Fotinos et al. 2008). Moreover, ALA is transported to cytosol via different active transport mechanisms. Even in Gram-negative bacteria, ALA is able to enter periplasm through the outer membrane porins and is transported to the cytosol by dipeptide permease (Elliott 1993). The synthesis of the porphyrins in the cytosol is strictly feedback controlled (Dietel et al. 2007).

This feature of micro-organisms looks very attractive, especially from the point of view of decontamination of food or food-related surfaces. Only living micro-organisms can metabolize colourless and odourless ALA to endogenous photosensitizers, which appears to be a way of gaining selectivity (Collaud et al. 2004). Meanwhile, no data exist on the evaluation of capacity of vegetative cells and spores of *B. cereus* to uptake ALA and to produce endogenous porphyrins. Moreover, there is no data on susceptibility of this bacterium to ALA-based photosensitization *in vitro* or on the different surfaces. Thus, the aim of this study was to focus on the possibility to inactivate vegetative forms and spores of food pathogen *B. cereus* by ALA-based photosensitization *in vitro* as well as on the packaging material.

Materials and methods

Chemicals

Stock solution of ALA (Fluka, Rehobot, Israel) was prepared by dissolving ALA in 100 mmol l⁻¹ phosphate-buffered saline (PBS, pH 7.2) up to the concentration of 200 mmol l⁻¹, and NaOH was used to adjust pH level of the solution to 7.2. ALA stock solutions were made instantly before use and sterilized by filtration through 0.20 µm filter (Roth, Karlsruhe, Germany) (Luksiene et al. 1999).

Bacterial growth

Bacillus cereus ATCC 12826 was kindly provided by the National Centre of Public Health (Vilnius, Lithuania). The bacterial culture was grown at 37°C and maintained on Luria Bertani agar (LBA; Liofilchem, Roseto degli Abruzzi, Italy).

The bacterial culture was grown overnight (c. 14 h) at 37°C in 20 ml of Luria-Bertani medium (LB) (Liofilchem), with aeration of 120 rev min⁻¹ (Environmental Shaker-Incubator, model ES-20; Biosan, Riga, Latvia). The overnight bacterial culture grown in LB medium was diluted 20 times with the fresh LB medium. The initial

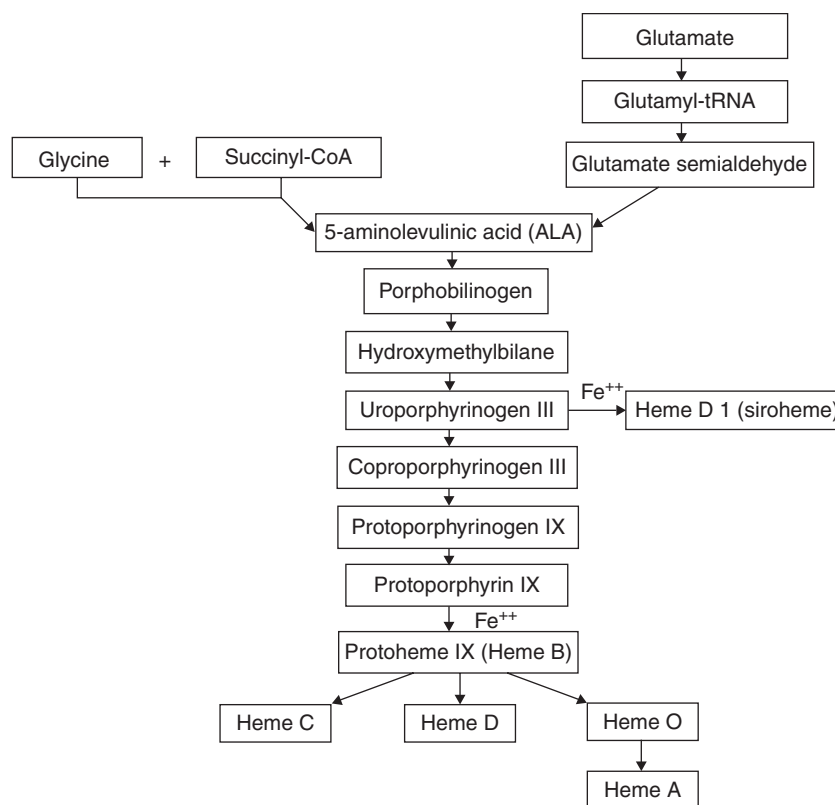


Figure 1 Bacterial biosynthetic pathway of heme (adapted from Hamblin and Hasan 2004). Synthesis of endogenous porphyrins from 5-aminolevulinic acid.

optical density (OD) of bacteria culture was 0.164 at $\lambda = 540$ nm (Helios Gamma and Delta spectrophotometers; ThermoSpectronic, Cambridge, UK). Bacteria were grown at 37°C to the midlog phase ($c. 6 \times 10^7$ CFU ml⁻¹, OD₅₄₀ = 1; bacterial count established by spread plate method) in a shaker (120 rev min⁻¹). Afterwards, the bacteria were harvested by centrifugation (10 min, 5000 g) and resuspended to $c. 3.015 \times 10^8$ CFU ml⁻¹ final concentration in 100 mmol l⁻¹ PBS (pH 7.2). This stock suspension was accordingly PBS-diluted to $c. 1 \times 10^7$ CFU ml⁻¹ and immediately used for the photosensitization experiments.

Photosensitization *in vitro*

Aliquots (10 ml) of bacterial suspension ($c. 1 \times 10^7$ CFU ml⁻¹ in 100 mmol l⁻¹ PBS buffer, pH 7.2) with appropriate concentrations of ALA (3 and 7.5 mmol l⁻¹) were incubated in the dark in a 50 ml plastic bottle for cell culture cultivation at 37°C. For the following experiments, the cells were incubated in the shaker (120 rev min⁻¹) for different periods (0–60 min). After incubation, 150 μ l aliquots of bacterial suspension were withdrawn, placed in sterile flat bottom wells and exposed to light for different periods (0–20 min) (Nitzan *et al.* 2004). Light emitting diodes (LED)-based light source (constructed in

the Institute of Applied Sciences of Vilnius University) for emitted light $\lambda = 400$ nm with the intensity of 20 mW cm⁻² on the surface of samples. Light dose was calculated as light intensity multiplied on time. Light power density measurements were observed with a light energy measure by 3 sigma meter (Coherent, Santa Clara, CA, USA) equipped with piro-electrical detector J25LP04. No thermal effects were detected at the exploited experimental conditions.

Fluorescence measurements of intracellular porphyrins

The cell suspensions for measurements of endogenous porphyrins from ALA were prepared as follows. Cells ($c. 1 \times 10^7$ CFU ml⁻¹ in 100 mmol l⁻¹ PBS, pH 7.2) were incubated in the dark at 37°C with 3 and 7.5 mmol l⁻¹ ALA concentrations for the indicated period. Then, 2 ml aliquots of bacterial suspension were withdrawn, resuspended in PBS and afterwards used for fluorescence measurements. PerkinElmer model LS-55 fluorescence spectrophotometer (Beaconsfield, UK) was used for the fluorescence detection. Scan range parameters are as follows:

- i Excitation wavelength – 390 nm
- ii Emission – 590–750 nm
- iii Excitation slit – 2.5 nm

- iv Emission slit – 15 nm
- v Scan speed (nm min⁻¹) – 200.

Evaluation of endogenous porphyrins produced by cells was performed according to the methodology described in our previous work (Luksiene et al. 2001). The fluorescence data were analysed with Origin 7.5 software (OriginLab Corporation, Northampton, MA, USA).

Photoinactivation of *Bacillus cereus* spores *in vitro*

For preparation of inocula of *B. cereus* ATCC 12826 spores, culture was grown for 3 days at 37°C in brain heart infusion broth (Liofilchem) containing (per litre) 0.05 mg manganese until 80–90% sporulation was obtained. Sporulation intensity was determined by Wirtz–Conklin staining method (Schaeffer and MacDonald 1933). Spore suspension was then washed three times by suspending the spores in 5 ml of sterile distilled water, centrifuging at 6000 g for 20 min and removing the supernatant. After washing, the spores were resuspended in 1 ml of distilled water, transferred to a sterile, plastic test tube and heated to 80°C for c. 15 min to inactivate any remaining vegetative cells. Aliquots (10 ml) of spore suspension (c. 1 × 10⁸ CFU ml⁻¹ in 100 mmol l⁻¹ PBS buffer, pH 7.2) with appropriate concentrations of ALA (3 and 7.5 mmol l⁻¹) were incubated in the dark in a 50 ml plastic bottle for cell culture cultivation at 37°C for 30 min; initial spore count was established by spread plate method. After incubation, 150 µl aliquots of bacterial suspension were withdrawn, placed in sterile flat bottom wells and exposed to light for 15 min. Afterwards, the purity of spores was tested by looking for the absence of vegetative cells using the Wirtz–Conklin staining method.

Photoinactivation of bacteria after adhesion to the surface of packaging material

Packaging yellow trays were provided by LINPAC (West Yorkshire, UK). In order to simplify experiments, the packaging samples for photosensitization experiments were cut into 4 × 8 cm pieces and each soaked in 50 ml *B. cereus* ATCC 12826 suspension (c. 1 × 10⁷ CFU ml⁻¹) for cell adhesion. Afterwards, the packaging samples were kept in laminar 30 min for further bacterial adhesion. Then, appropriate packaging samples were incubated in the dark with the 3 mmol l⁻¹ concentration of ALA for different periods (5, 10 and 20 min). The control samples were incubated with PBS (7.2 pH).

After incubation with ALA, all packaging samples were dried in a laminar at room temperature for 20 min. Dried samples were placed in the treatment

chamber and exposed to light for different time (5, 10, 15 and 20 min). The control sample was not illuminated.

Photoinactivation of *Bacillus cereus* spores after adhesion to the surface of packaging material

Bacillus cereus spore suspension was prepared as described previously. Packaging samples (2.5 × 4 cm) were placed in sterile plastic tubes, and spore solution (c. 1 × 10⁸ CFU ml⁻¹) was added until all samples were completely submerged for 30 min at 37°C. Afterwards, coupons were withdrawn and kept in laminar 30 min for further spore adhesion. Then, samples were placed in plastic tubes containing 25 ml of ALA solution with the concentration of 3–7.5 mmol l⁻¹ and incubated in the dark for 30 min. The control coupons were incubated with sterile PBS. After incubation with ALA, all packaging samples were dried in laminar at room temperature for 20 min. Dried samples were placed in the treatment chamber and exposed to light for 15 min. The control samples were not illuminated.

Bacterial cell survival assay

The antibacterial effect of photosensitization on *B. cereus* was evaluated by the spread plate method. Thus, 100 µl of appropriate dilutions of bacterial test culture after photosensitization *in vitro*, using the spread plate method, was surface inoculated on the separate LBA plates. Afterwards, the bacteria were kept in the incubator for 24 h at 37°C. The surviving cell populations were enumerated and expressed as N/N_0 , where N_0 is the number of CFU ml⁻¹ in the untreated culture and N is the number of CFU ml⁻¹ in the treated culture.

After treatment, each packaging sample was mixed with 30 ml of 100 mmol l⁻¹ PBS buffer and homogenized with a BagMixer (model MiniMix 100 VP; Interscience, St Nom, France). Then, 100 µl of appropriate dilutions (0.9% NaCl) of suspension was placed on LBA plates. The colonies were counted after 24 h incubation at 37°C. The surviving cell populations were enumerated and expressed as log₁₀ (CFU ml⁻¹ and CFU cm⁻¹).

Statistical analysis

The experiments were carried out in triplicate for each set of exposure. A standard error was estimated for every experimental point and marked in a figure as an error bar. Sometimes the bars were too small to be visible. The data were analysed with Origin 7.5 software (OriginLab Corporation).

Results

Detection of endogenous porphyrins synthesized from ALA in *Bacillus cereus* cells

In fact, not every bacterium can synthesize endogenous porphyrins from ALA in the amounts necessary for photosensitization-based inactivation (Fotinos *et al.* 2008). Thus, in the first stages of this study, it was necessary to evaluate the potential of Gram-positive bacterium *B. cereus* to produce endogenous porphyrins from extrinsically applied ALA. According to Szocs *et al.* (1999), the spectral region with maximum at $\lambda = 612\text{--}615\text{ nm}$ is attributed to the presence of endogenously synthesized porphyrins including uroporphyrins and coproporphyrins. For this purpose, fluorescence emission spectra in the region of 590–680 nm were analysed after incubation of cells with 3–7.5 mmol l⁻¹ ALA in the dark. Figure 2(a) shows the excitation spectrum of endogenous porphyrins produced by *B. cereus* after incubation with 3 mmol l⁻¹ ALA. Thus, the following fluorimetric analysis indicated that the intensity of fluorescence emission of endogenous porphyrins after incubation of cells with 3 mmol l⁻¹ ALA for 0–60 min was increasing in a time-dependent manner (Fig. 2b). Fluorescence intensity of endogenous porphyrins was insignificant after 2 min of incubation with ALA but increased and reached more than 500 fluorescence intensity units after 30–60 min incubation. In order to understand whether a suitable concentration of ALA solution was used, we increased ALA concentration to 7.5 mmol l⁻¹. Analysis of the fluorescence emission spectra of endogenous photosensitizers indicated that there was some increase (up to 650 units) in fluorescence intensity of endogenous porphyrins when the same incubation time (60 min) was used (Fig. 2c).

Inactivation of *Bacillus cereus* by ALA-based photosensitization

Analysis of fluorescence emission spectra of endogenous porphyrins indicates that this Gram-positive bacterium can effectively synthesize endogenous photosensitizers from ALA. It means that the main premise for the possibility to inactivate the troublesome food pathogen by ALA-based photosensitization was identified. Thus, the obtained results have prompted us to evaluate the applicability of ALA-based photosensitization for the inactivation of *B. cereus*. The data obtained revealed that toxicity of ALA alone (3–7.5 mmol l⁻¹), without illumination, on viability of *B. cereus* was, as usual, negligible. The following illumination of bacterial cells (0–20 min) decreased the survival fraction fairly sharply (Fig. 3a,b). It is important to note that a very short illumination time of

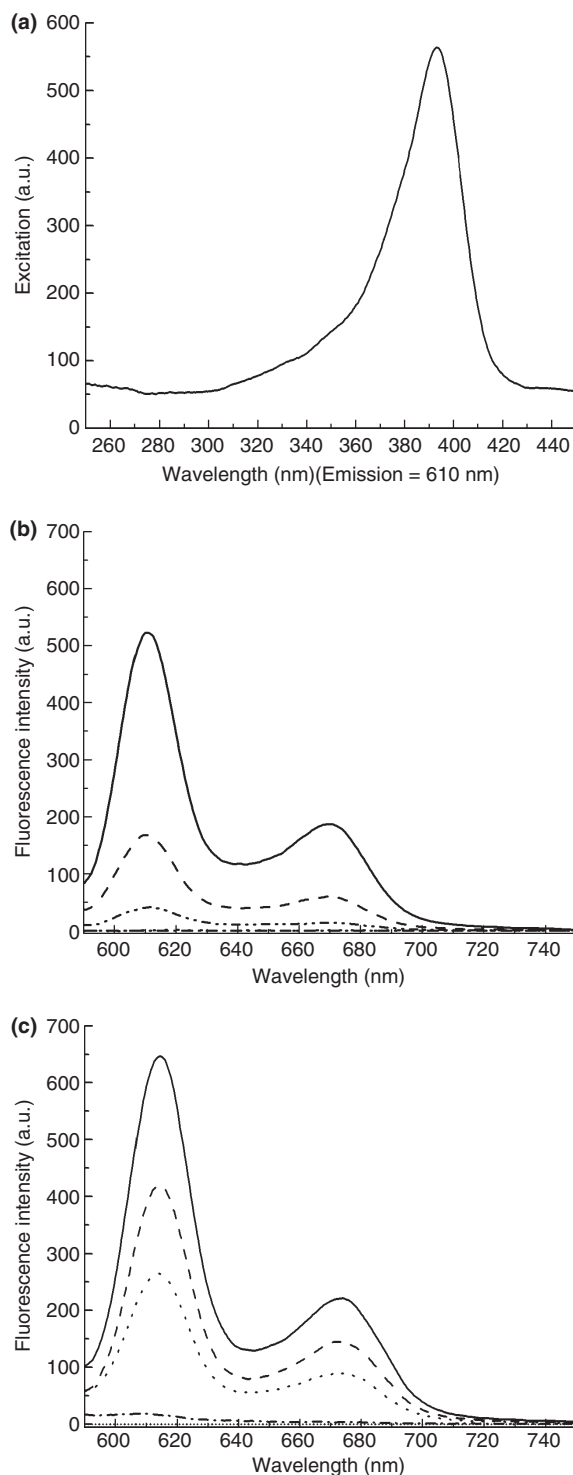


Figure 2 Excitation spectrum of endogenous porphyrins produced by *Bacillus cereus* after incubation with 3 mmol l⁻¹ 5-aminolevulinic acid (ALA) (a); fluorescence spectra of endogenous porphyrins produced by *B. cereus* after incubation with 3 mmol l⁻¹ ALA for different time intervals (b); fluorescence spectra of endogenous porphyrins produced by *B. cereus* after incubation with 7.5 mmol l⁻¹ ALA for different time intervals (c).

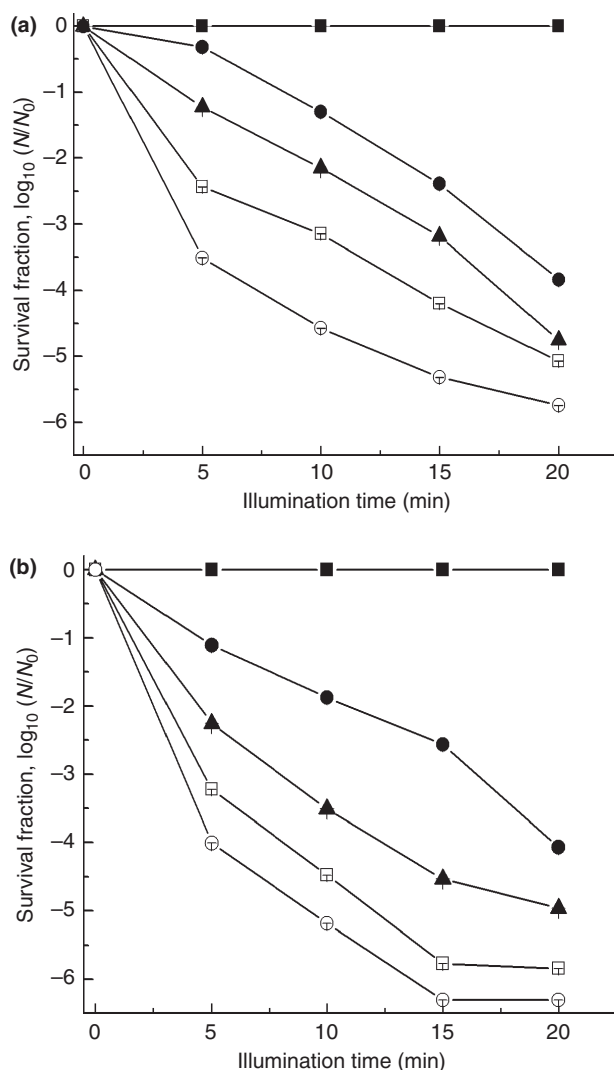


Figure 3 Inactivation of *Bacillus cereus* by 3–7.5 mmol l⁻¹ 5-aminolevulinic acid (ALA)-based photosensitization as function of illumination time (a, b); bacteria were incubated with ALA solution for different periods (2–60 min): (■) control; (●) 2 min; (▲) 15 min; (□) 30 min and (○) 60 min. Every point is the average of 3–6 experiments, and error bars sometimes are too small to be more visible.

0–5 min was not effective, as not all endogenous porphyrins were excited by light to S₁ to produce cytotoxic reactions because of the lack of photons delivered during short time.

From the data presented in Fig. 3a, it is clear that the prolongation of period of incubation (from 2 to 60 min) with 3 mmol l⁻¹ ALA increased the rate of bacterial inactivation. The use of 60 min incubation time and 20 min illumination time resulted in 5.75-log inactivation of *B. cereus*. As a matter of fact, photosensitization efficiency can be expressed as a function of accumulated photosensitizer multiplied on the total light energy delivered to

the bacterial cells. Taking this into account, we investigated the inactivation of *B. cereus* by ALA-based photosensitization as a function of time, using higher ALA concentration (7.5 mmol l⁻¹). Data presented in Fig. 3b reveal that higher ALA concentration (7.5 mmol l⁻¹) can enhance the inactivation of *B. cereus*. This inactivation of bacteria strongly depends on the period of incubation with ALA as well. A short incubation time (2 min) is not enough to produce endogenous porphyrins from ALA, whereas 15–20 min incubation time is much more suitable for the development of perspective antibacterial technology.

Inactivation of *Bacillus cereus* spores by ALA-based photosensitization

In the next step, sensitivity of *B. cereus* spores to ALA-based photosensitization was tested. According to Demidova and Hamblin (2005), shorter incubation time and lower temperature are less effective, suggesting that photosensitizer that penetrates into the spores by passive diffusion process data, presented in Fig. 4, indicated that the incubation of spores with ALA (30 min) did not induce significant changes in their number. Following illumination (15 min) of spores can drastically reduce their population from 8 log in the control to 4.3 log in the treated samples. The inactivation rate of spores, as usually, strongly depended on mentioned ALA concentration. For instance, the use of 3 mmol l⁻¹ ALA concentration at given experimental conditions can inactivate spores about 2 log, whereas 7.5 mmol l⁻¹ ALA concentration inactivates spores by 3.7 log.

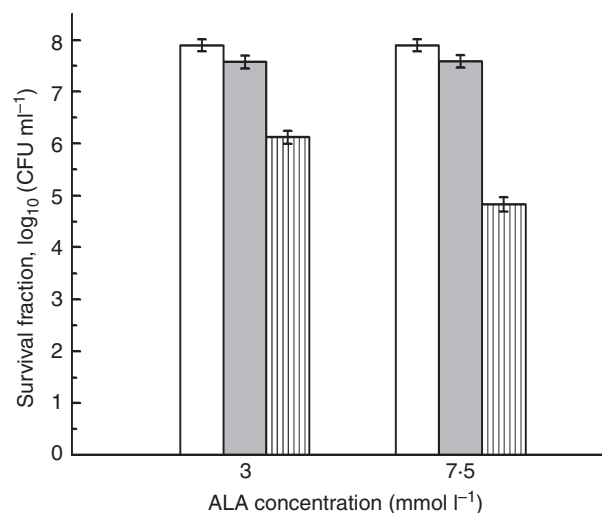


Figure 4 Inactivation of *Bacillus cereus* spores *in vitro* by 5-aminolevulinic acid (ALA)-based photosensitization: (□) control; (■) ALA without light and (▨) ALA + 15 min light.

Decontamination of food-related packaging from *Bacillus cereus* by ALA-based photosensitization

In order to evaluate antibacterial efficiency of ALA-based photosensitization for decontamination of surface, *B. cereus* was attached to food packaging material (yellow trays) provided by LINPAC. Packaging samples (10 × 10 cm) after soaking in ALA solution (3 mmol l⁻¹) for 10 min were illuminated 5–20 min with 405 nm, 20 mW cm⁻² light. Afterwards, treatment-survived bacteria were counted. Data, depicted in Fig. 5a, describe the ALA-

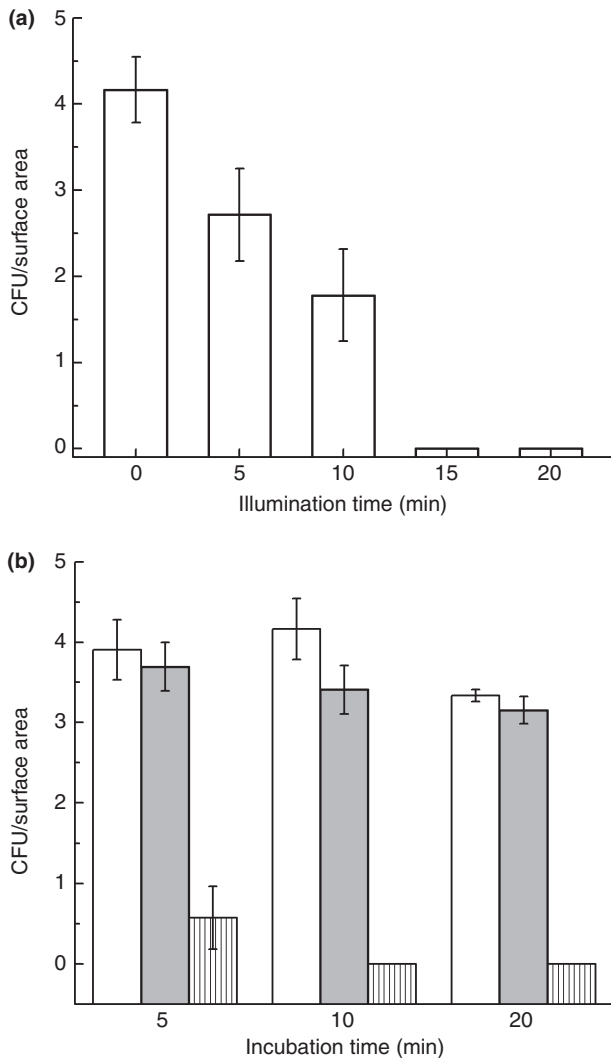


Figure 5 (a, b) Inactivation of *Bacillus cereus* ATCC 12826 attached on packaging samples by 3 mmol l⁻¹ 5-aminolevulinic acid (ALA)-based photosensitization as function of illumination time. Incubation time with ALA was 10 min. (a) Inactivation of *Bacillus cereus* by 3 mmol l⁻¹ ALA-based photosensitization onto packaging samples as function of incubation time. (b) Illumination time was 20 min: (□) control; (■) 3 mmol l⁻¹ ALA without light and (▨) 3 mmol l⁻¹ ALA + 20 min light.

based photoinactivation of *B. cereus* as a function of illumination time. It is clear that 20 min illumination is more than enough to inactivate *B. cereus* by 4 log, when the period of incubation with ALA is 10 min. Results, presented in Fig. 5b indicate, that antibacterial efficiency of ALA-based photosensitization is a function of the period of incubation with ALA as well. When the period of incubation with ALA is short (5 min), the production of endogenous photosensitizers in bacteria is insufficient, and consequently inactivation of *Bacillus* reached just 3·3 log. Further prolongation of the period of incubation with ALA up to 10 or 20 min increases the antibacterial efficiency up to 4 log. Results, depicted in Fig. 5a,b, indicated that selected experimental parameters (period of incubation with ALA, illumination time) were optimal to decontaminate packaging materials from *B. cereus* at certain experimental conditions.

Decontamination of food-related packaging from *Bacillus cereus* spores by ALA-based photosensitization

In order to estimate the decontamination efficiency of ALA-based photosensitization, food packaging material was submerged in *B. cereus* spore solution. Different concentrations of ALA solution (3 and 7·5 mmol l⁻¹) were exploited for experiments. Illumination parameters were as follows: λ = 405 nm, 20 mW cm⁻² and 15 min. Data, shown in Fig. 6, indicate that *B. cereus* spores are able to attach to plastic food-related packaging material and can be inactivated by ALA-based photosensitization as well as *in vitro* experiments. ALA itself only slightly

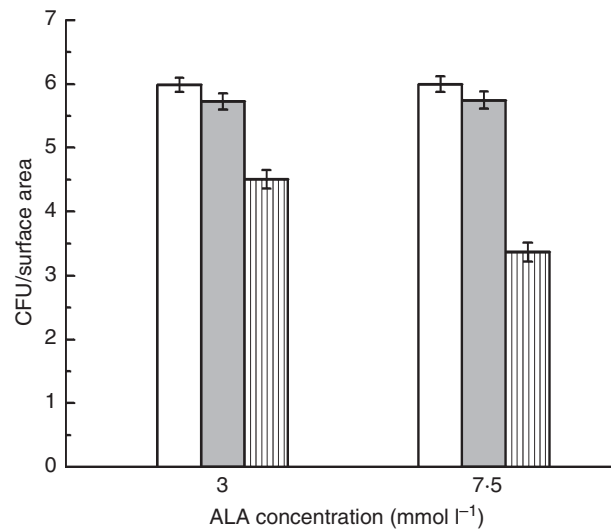


Figure 6 Decontamination of food-related packaging from *Bacillus cereus* spores: (□) control; (■) 5-aminolevulinic acid (ALA) without light and (▨) ALA + 15 min light.

influences spores inactivation. After 30 min incubation, only 0.3-log decrease is received, whereas 15 min light exposure ensures 1.5- and 2.7-log reductions.

Discussion

Antimicrobial photosensitization-based technique has been developed especially during the last 25 years (Jori 2006). The main prerequisite for the rise was the growing concerns about the emergence of antibiotic-resistant bacterial strains. It is worth emphasizing that the phototoxic action on pathogens is caused by the combined action of two factors: photosensitizer and visible light that separately are devoid of any detectable toxicity at used doses (Jori 2006).

One of the most important advantages of photosensitization in comparison with other antibacterial tools is the absence of any bacterial resistance to this treatment (Nitzan and Ashkenazi 2001), because this process is free radical mediated (Polio et al. 2002). Moreover, because of the fact that a photoactive compound is localized inside micro-organisms, all cytotoxic reactions are just local. It allows us to achieve an extensive decrease in the population of pathogens with minimal damage effects on the surrounding matrix (Jori 2006). Eventually, it was confirmed that photosensitization, in contrast to ionizing radiation, has no mutagenic or carcinogenic effects on living systems (Luksiene 2005; Luksiene et al. 2006). ALA-based photosensitization was found to induce a more pronounced antibacterial effect on Gram-positive bacteria than on Gram-negative ones. Nitzan et al. (2004) reported that ALA and 50 J cm⁻² light intensity resulted in five orders of decrease in *Staphylococcus* strains as opposed to 1–2 orders of decrease in Gram-negative strains.

The data obtained in this study clearly indicate that Gram-positive *B. cereus* can effectively produce endogenous photosensitizers from exogenously applied ALA even at 3 mmol l⁻¹ concentration. It was shown in our previous studies that Gram-negative food pathogen *Salmonella enterica* as well as Gram-positive pathogen *L. monocytogenes* can produce endogenous porphyrins in amounts suitable for effective photosensitization just at 7.5 mmol l⁻¹ ALA concentration. A comparative analysis of fluorescence intensity of endogenous photosensitizers reveals that *B. cereus*, during 60 min incubation with ALA, is producing endogenous porphyrins ten times more efficient than *L. monocytogenes* or *Salm. enterica* (Buchovec et al. in press). As a consequence, *B. cereus* is more susceptible to ALA-based photosensitization than *Salm. enterica* or *L. monocytogenes*. Really fast and significant inactivation (6.5 log) of this micro-organism can be achieved after ALA-based photosensitization, whereas

most resistant biofilm forming food pathogen *L. monocytogenes* after 2 h incubation with ALA and 20 min illumination was inactivated by four orders of magnitude (Buchovec et al. in press).

Because of very high resistance of bacterial spores to UV (7–50 times more resistant than vegetative cells) (Nicholson et al. 2000), germicidal lamps are insufficient to ensure sterilization of surfaces, including packaging materials. More than 90% of packaging contamination is composed of aerobic, spore-forming bacteria (Pirttijarvi et al. 1996). In some cases, hydrogen peroxide solution (35% v/v) is sprayed onto the surface of packaging and subsequently removed by the stream of hot sterile air (Holdsworth 1992). Hence, there is a recognized risk of chemical food contamination by residues of hydrogen peroxide, relatively high concentration of this compound is employed.

Decontamination of packaging material from *B. cereus* adhered to the surface by this treatment seems really promising. More than 4-log inactivation was achieved after ALA-based photosensitization. Moreover, obtained data indicated that the *Bacillus* spores are susceptible to this treatment as well. Even 3.1-log reduction in spore population was observed after ALA-based photosensitization *in vitro* and 2.7 log on the surface of packaging material.

Decontaminating food or food-related surfaces by photosensitization is novel and pioneering approach. Undoubtedly, the main premise in this case is the used photosensitizer, which besides its suitable photophysical and photochemical properties must be a pure and water soluble compound with a stable shelf-life, not bleaching and easy to produce. Moreover, it must be food constituent or food additive. When colourless and odourless ALA is used, just living micro-organisms on the matrix surface can metabolize it to endogenous porphyrins that act as photosensitizers. Thus, this appears to be a way of gaining selectivity. After illumination of such surface with bacteria, reactive oxygen species can be selectively generated within the microbes without oxidative damage to food constituents and unwanted colouration. Moreover, other question 'how does ALA interact with food matrix' arises. Our previous experiments on the decontamination of wheat sprouts by ALA-based photosensitization indicate that ALA stimulates the growth of wheat seedlings and roots without impairing the vigor of germination and the viability of seeds. Moreover, ALA increases the rate of photosynthesis and the activities of antioxidant enzymes, what can be associated with enhanced cellular capacity to detoxify reactive oxygen species (Luksiene et al. 2007). Moreover, ALA is an essential precursor of such tetrapyrrole compounds like vitamin B12 and hemes, which serve as prosthetic groups of respiratory enzymes and

chlorophyll in plants (Granick 1961). Suitable ALA concentrations have promotive effects on the growth rates. Crop yields were enhanced by the application of ALA at the leaf-stage for rice, barley, potato and garlic (Tanaka et al. 1992). Foliar application of ALA (100 mg ml⁻¹) on date palm has promoting effects on the fruit weight, volume and sugar content (Hotta et al. 2002).

Conclusions

The data obtained in this work clearly indicated that *B. cereus* can effectively produce endogenous photosensitizers from exogenously applied ALA even at very low concentrations (3 mmol l⁻¹). Really significant inactivation (6.3 log) of this micro-organism can be achieved after ALA-based photosensitization *in vitro*. The decontamination of packaging material from adhered *B. cereus* after ALA-based photosensitization reached 4 log. Of importance to note, that spores of *B. cereus* are susceptible to this treatment and can be inactivated by 3.1 log *in vitro* or 2.7 log on the surface of packaging material.

In conclusion, our previous data, as well as the one obtained in this study, support the idea that photosensitization is not 'magic bullet' against all pathogens, but its high selectivity, antimicrobial efficiency and nonthermal nature, may be, will serve in the future for the development of completely safe, nonthermal surface decontamination and food preservation technique.

Acknowledgement

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