Biotoxicity evaluation of singlet oxygen generated by immobilized porphyrin

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Introduction

Many light-activated antimicrobial materials have been so far prepared with potential application to medicine, biotechnology and food industry (Artarsky et al., 2006; Mosinger et al., 2007 and Savino et al., 1985). Their antimicrobial effect is based on photogeneration of singlet oxygen by photosensitive dyes immobilized in polymers.

Singlet oxygen generated by photosensitive reaction enters the microbial cell and can react with other molecules to form reactive oxygen species (ROS) such as oxygen ions, free radicals, and inorganic and/or organic peroxides. Reactions of ROS with some biomolecules (for example DNA, lipids or proteins) lead to cytotoxic events. Eventually, the target cell is killed by apoptosis or necrosis (Luksiene, 2005).

Bacteria built up mechanisms to protect themselves against oxidative stress, utilizing enzymes such as catalase and superoxide dismutase or carotenoids production (Cabiscol et al., 2000). Cell wall architecture is also important in this respect. Gram-positive bacteria have been found more sensitive to photosensitization than Gram-negative bacteria (Luksiene, 2005).

Because a convenient and cheap microbiological method for testing toxicity of photoactive polymers is still lacking, in this work, three Gram-positive bacterial strains (*Bacillus amyloliquefaciens* 3129, *Lactobacillus helveticus* CH-1, and *Rhodococcus* sp.) were applied to develop new microbiological experimental methods of Visual Image Analysis (VIA). A photosensitive layer prepared by entrapment of porphyrin TMPyP into a silica matrix prepared from TMOS was used as a model photosensitive polymer.

Material and methods

Chemicals, strains and microbiological media

Chemicals:
Tetramethoxysilane (TMOS) and 5,10,15,20-*tetrakis*(1-methyl-4-pyridinio)porphyrin tetra(*p*-toluenesulfonate) (TMPyP) were purchased from Fluka (Switzerland). NaOH, HCl, NaCl, I_2, KI, glucose, and starch were obtained from Lach-Ner (CZ). MRS agar, MRS broth and tryptone were purchased from Oxoid (UK). Yeast extract was purchased from AppliChem (Germany).

Microorganisms:
Selected bacterial strains of *Lactobacillus helveticus* CH-1 (*Lbc.*), *Bacillus amyloliquefaciens* 3129 (*Bc.*) and *Rhodococcus* sp. (*Rhod.*) were obtained from FFBT ICT Prague (CZ) – see Tab. 1.

Microbiological media:
LB medium: NaCl (10 g), tryptone (10 g), yeast extract (5 g), distilled water (1000 mL), resp. agar (20 g).

Starch agar: tryptone (5 g), yeast extract (2.5 g), glucose (1 g), starch (10 g), agar (20 g), and distilled water (1000 mL).
Lugol solution: KI (2 g), I_2 (1 g), and distilled water (300 mL).

	Gram	Metabol.	Spor.	Shape	CAT	OX	SOD	MnPP	G-C	Carot.
Lbc.	+	microaer.	-	rod	-	-	-	+	low	-
Bc.		aerobic	+		+	+	+	-	high	
Rhod.			-	rods or cocci in mycelium						+

Table 1: Bacterial strains characterizations. Legend: Spor. – sporulation, Metabol. – metabolism, CAT – catalase, OX – cytochrome c oxidase, SOD – superoxide dismutase, MnPP – manganese polyphosphate, G-C – guanine-cytosine content, microaer. – microaerophilic, Carot. - carotenoids.

TMOS prepolymerization
TMOS was mixed with deionized water and HCl in 51.3:25:6.25 volume (mL) ratio to form a clear solution and left to prepolymerize at 4°C for 16 h.

Layers preparation
TMOS prepolymer was mixed with TMPyP solution in deionized water (10^{-4} M) and NaOH solution (0.05 M) in 1:1:0.5 volume (mL) ratio. The layers were prepared by pouring the mixture onto a glass Petri dish (ø 3.5 cm). After gelation (c. 2 min), the TMOS layers poured over by deionized water were stored in the dark at 4°C. Positive control was made by mixing of TMOS prepolymer with deionized water without dissolved porphyrin.

Antimicrobial activity test
Conditions of antimicrobial activity test of TMOS-TMPyP layer for each strain are given in Tab. 1.

	Liq. medium	Agar	Incubation	Petri dish treatment	Evaluation
Lbc.	MRS at 42°C*	MRS	42°C for 72 h	-	light colonies
Bc.	LB at 30°C	Starch	30°C for 48 h	0.25 mL Lugol solution	light agar
Rhod.	LB at 30°C	LB	30°C for 96 h	-	pink colonies

Table 2: Conditions of antimicrobial activity test. * Anaerobic conditions.

Bacterial strains were cultivated in liquid medium at their optimal growth temperature for 16 h. Each inoculum was diluted to the concentration of 10^4 CFU/mL into agar. The inoculated agar (1.5 mL) was poured over a TMOS layer at the bottom of a Petri dish. Negative control was made by pouring the inoculated agar at the bottom of an empty Petri dish without TMOS layer. The Petri dishes were illuminated by 300W halogen lamp from distance of 70 cm for 0; 1.5, and 3 h, resp. Illuminance (lx) was measured with Lutron LX-103 light meter. After illumination, the inoculated Petri dishes were incubated in the dark at optimal growth temperature. Then, the images of Petri dishes were taken and percentage bacterial growth on Petri dishes was evaluated using NIS Elements BR 2.30 software.

Results and Discussion

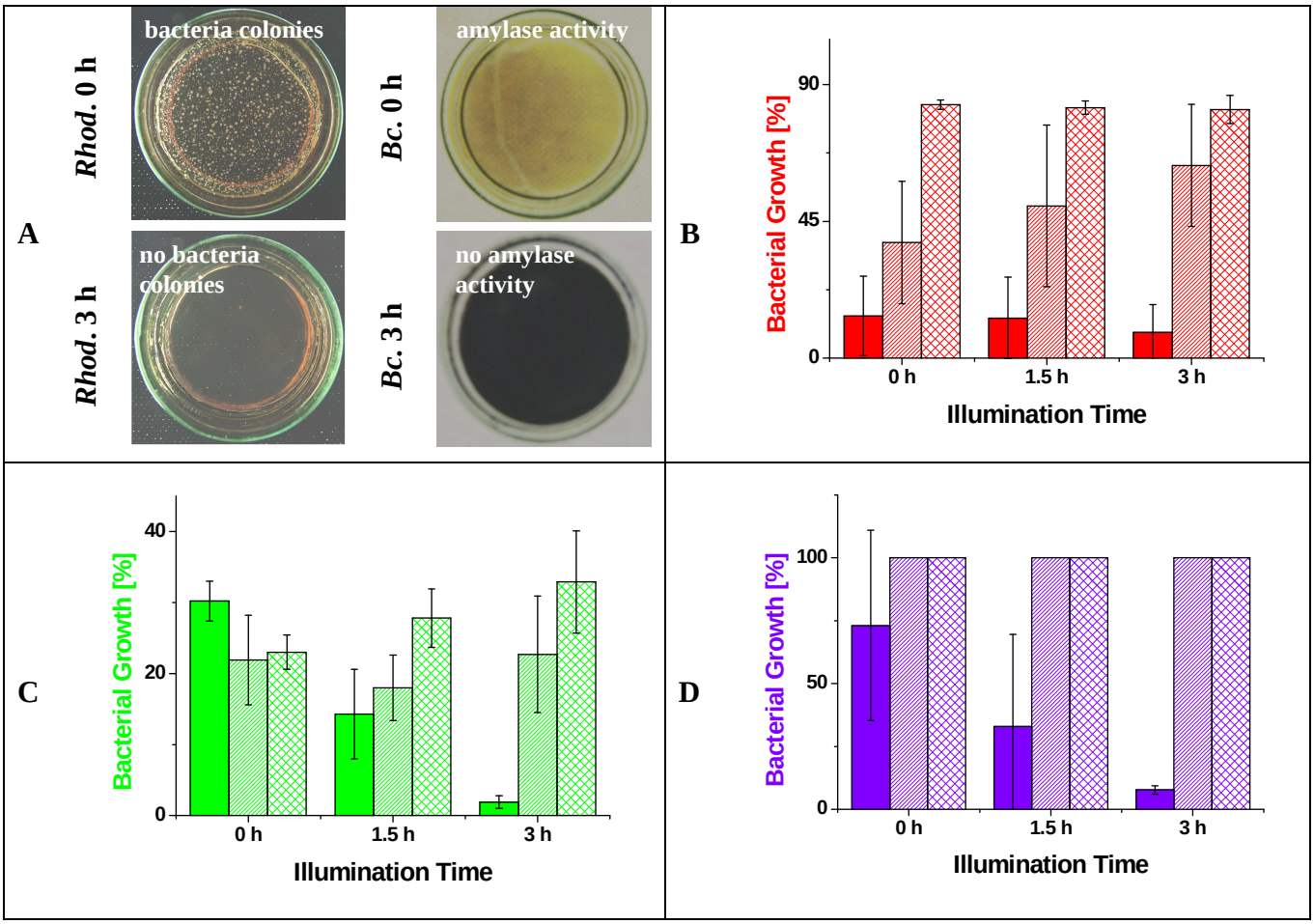

Figure 1: Biotoxicity evaluation of singlet oxygen using VIA. Legend: A – images of Petri dishes with *Rhodococcus* sp. and *Bc. amyloliquefaciens* after illumination of 0 and 3 h; VIA evaluation: B – *Lbc. helveticus*; C – *Rhodococcus* sp.; D – *Bc. amyloliquefaciens*;
■ TMOS with TMPyP , ▨ positive control - TMOS without TMPyP, ⊠ negative control - no TMOS

Image analysis systems are routine in many fields of science, and their applications in microbiology are numerous. In this work, new methods of Visual Image Analysis (VIA) for testing cytotoxicity of photoactive polymers were developed. For this reason, a stable photoactive TMOS-TMPyP layer and three bacterial genera, whose growth on agar is well eye-visible, were chosen (see Tab. 1 and 2). *Lbc. helveticus* CH-1 strain is a homofermentative bacterium able to form light colonies on dark MRS agar. *Rhodococcus* sp. is a member of phylum *Actinobacteria* and contains pink carotenoids in its cell wall. These two strains were evaluated using the method which directly counts the growth of colonies on agar. *Bc. amyloliquefaciens* 3129 strain is a microorganism with a strong amylase activity. That is why the indirect method using Lugol solution coloration of starch agar was used to evaluate its growth on Petri dishes. All the strains chosen are Gram-positive.

For all three types of experiments, the values of incident luminous energy on the Petri dishes during illumination process were about 30; 30,000 and 60,000 lm.s for illumination time of 0, 1.5 and 3 h, resp. The results of biotoxicity tests are shown in Fig. 1. In contrast to the other two strains,

the immediate cytotoxic effect of photoactive layer was observed for *Lbc. helveticus* which is microaerophilic and has not built up a strong enzymatic antioxidative system such as superoxide dismutase or catalase. Lactobacilli accumulate only millimolar quantities of manganese polyphosphate for protecting the cells from oxygen damage (en.wikipedia.org). During the experiment, bacterial growth of *Rhodococcus* sp. on Petri dishes with photosensitive layers gradually decreased from 30 % to 2 %. In comparison to direct *Lbc.* experiment, the decreasing singlet oxygen sensitivity is likely due to aerobic metabolism related to a strong enzymatic detoxication system and protective effect of carotenoids. An indirect method using amylase activity test was used for *Bc. amyloliquefaciens*. Whereas amylase activity of *Bc. amyloliquefaciens* on both positive and negative control was evaluated as 100 % during the whole experiment, the average starting amylase activity on TMOS-TMPyP layers was about 60 % and decreased by about 55 % during illumination time of 3 h. Generally, the bactericidal effect of positive control of TMOS without porphyrins was observed against negative control. This effect is probably due to methanol release during TMOS polymerization.

Summarizing, of the methods tested, the direct *Lbc*.-method seems to be the more sensitive, whereas the direct *Rhod*.-method is better visible and easier to evaluate. The indirect *Bc*.-method is the least time-consuming but not very sensitive.

Conclusions

Bacterial strains of *Bacillus amyloliquefaciens* 3129, *Lactobacillus helveticus* CH-1 and *Rhodococcus* sp. were applied to develop singlet oxygen biocide tests using the VIA method. In comparison to frequently used microbiological methods for determination of photoactive polymers toxicity (see References), the present methods are relatively easy, cheap and more convenient and do not use GMOs as testing microorganisms.

References

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