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The efficacy of photodynamic inactivation of the microorganisms using laser sources and methylene blue as sensitizer

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The large spread of pathogen microorganisms in nature and their increasing resistence to drugs, lead to alternative treatment methods, more efficient and without microbial resistance. One of these methods is the photodynamic inactivation of microorganisms. In the present paper, we are discussing the efficacity of the laser system SCL (INOE 2000, P = 15 mW, λ = 635 nm) on yeast cells (*Saccharomyces cerevisiae* - as model system) treated with methylene blue as sensitizer. Diffuse reflectance spectroscopy and Kramers – Kronig analysis have been used for *in vitro* determination of the refractive index changes of yeast cells induced by laser irradiation.

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1. Introduction

Photodynamic inactivation (PDI) is a new alternative method for eradication of antibiotic resistance pathogenic microbes. This method is based on the concept that a photosensitizer is localized preferentially in the microorganism and subsequently activated by light of appropriate wavelength to generate reactive oxygen species which produce cell damages and inactivate the microorganisms [1].

Photodynamic inactivation of the microorganisms is dating from antiquity as it has been mentioned in ancient Egyptian medicine [2,3] where is mentioned the application of some plants called *babichi* (psoralea carylifolia) followed by sun exposure for the treatment of depigmented skin lesions (vitiligo). This plant contains psoralene, which is an anaerobic photosensitizer. In present, psoralene based drugs are used for the treatment of vitiligo and psoriasis (treatment based on the photochemical reaction without oxygen presence). The photochemical reaction oxygen dependent was discovered in 1898 by the student Oscar Raab who demonstrated the effect of light and some dyes on paramecia [4]. His experiments were done in Herman von Tappeiner laboratories in Munich. Herman von Tappeiner has continued the experiments and has introduced together Joldbauer in 1904 the term of *photodynamic reaction* as a concerned action of light, the photosensitizer and oxygen [5]. Since 1904 a lot of researchers have developed this therapeutic method demonstrating its value in the treatment of cancer and other diseases [6 – 15].

In the latest period, the attention of researchers was focused on the application of this method for inactivation of some microorganisms especially those with antibiotic resistance. Although only experimental stages are known up to now, there are remarkable results in killing by photodynamic inactivation of germs, which generate several types of infections [16-20].

For future improvement of the efficacy of photodynamic inactivation of clinically relevant microbes we sought to understand the influence of the irradiation parameters on the yeast cells such as *Saccharomyces cerevisiae* exposed to PDI using diffuse reflectance spectrometry. We have chosen the yeast as a model organism because they are easy to cultivate and can be used for research that is too complicated to be performed with higher cells. The same model organism was used by other researchers to assess the cell damage induced by PDI in eukaryotic cells [21-25].

Some photosensitizers and both coherent (lasers) and non-coherent (lamps and LEDs) light sources have been used for photodynamic inactivation of microorganisms, until now. The photosensitizers applied are known to act via cell membrane damage (methylene blue [26] and toluidine blue [27]) or via DNA modification causing genotoxic effects (8-methoxypsoralen [28]). Both types of light sources have their own advantages and disadvantages [29].

The aim of this paper is to study the efficacity of the laser system SCL (INOE 2000) on microorganisms like *Saccharomyces cerevisiae* - as model system using methylene blue (MB) as photosensitizer.

2. Materials and methods

2.1 Photosensitizers

Methylene blue (Aldrich), as analytical grade reagent, was used after laboratory further purification at ICECHIM, Bucharest. For our experiments was very important to remove aggregated Methylene blue (MB) forms from the system. Water redistilled from alkaline permanganate was used to prepare all solutions. MB was stored as aqueous stock solutions for a maximum of 2 weeks (10^{-5}M) at 4^{O} C in the dark before use.

For photodynamic inactivation tests, there has been used the solution with the concentration: C_{MB} = 1,08x10^{-5}M and pH = 7.4, in order to prevent the aggregation process.

2.2 Light sources

The illumination was carried out using a laser system SCL (INOE 2000, Bucharest, Romania) with power 15 mW and emitting at wavelength λ = 635 nm.

2.3 Yeast strain and growth conditions

The microorganism used in this study was *Saccharomyces cerevisiae* strain (SC-132). Cells were grown in liquid YPD (yeast peptone with 0.1 % dextrose) medium with constant agitation (300 rpm) at 30^{O} C. After 3 days (stationary phase) cultures were harvested, washed twice with 50 mM phosphate buffer (pH 6.8) and portions were resuspended (at 1x10^{7} colony forming units per ml) in either buffer alone (control lot) or in buffer containing sensitizer (three experimental lots vs. exposure time: 15 min, 20 min and 30 min).

2.4 Photodynamic inactivation studies

To investigate the photodynamic inactivation of the yeast, 10 µl of methylene blue was added to each well and the plates were incubated in the dark for 15 min at 37°C. The cultures were irradiated with a 635 nm wavelength of light using laser system SCL at different light doses: 4,328 J/cm^2, 8,656 J/cm^2 and 12,983 J/cm^2. The exposure time was: 15 min, 20 min and 30 min respectively. The light source was placed 1 cm over the bacterial suspensions (Fig. 1).

Fig. 1. Culture Saccharomyces cerevisiae cells irradiation

2.5 Diffuse reflectance spectrometry

The evaluation of the efficacy of photodynamic inactivation of the *Saccharomyces cerevisiae* cells was done using the diffuse reflectance spectrometry (fig. 2). The optical reflectance spectra of the Saccharomyces Cerevisiae cells were obtained with AvaSpec 2048 spectrometer (Avantes) in wavelength range (500 – 1100) nm before and after each laser irradiation and the variation of the refractive index was determined with Kramers-Kronig analysis of the reflectance spectra.

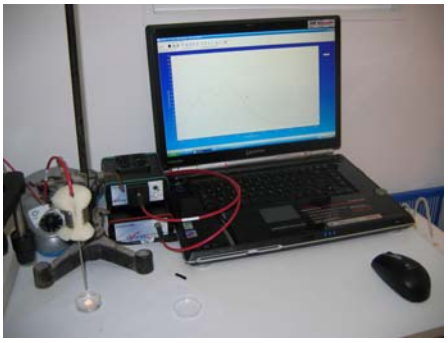


Fig. 2. The experimental set-up for diffuse reflectance measurements

2.6 Microscopy

Combined differential interference contrast was used to visualize the shape of individual yeast cells. The microscope was equipped with a videocamera (Sony), a video recorder and a dual image processor (Hammamatsu).

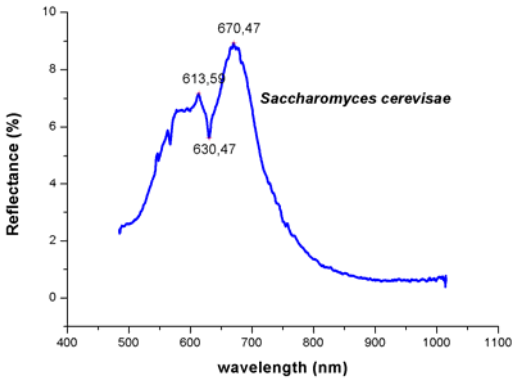
2.7 Statistical methods

Data are presented as means ±SD. The mean value and its standard deviation were calculated using Microsoft Excel.

3. Results and discussion

The diffuse reflectance spectrum of *Saccharomyces cerevisiae* culture presents two main reflectance maxima, at $\lambda_{1\text{-max}}$ = 610.56 nm and $\lambda_{2\text{-max}}$ = 670.47 nm, and a minimum at λ_{min} = 630.47 nm (fig. 1.a). When the *Saccharomyces cerevisiae* culture is incubated with MB for 15 min in the dark, the reflectance spectrum presents some changes due to absorption characteristics of MB showing three reflection maxima: λ_1 = 610.87 nm λ_2 = 670.19 nm and λ_3= 734.48 nm (fig. 1.b). The *Saccharomyces cerevisiae* culture with MB as added photosensitizer shows a hypsochromic reflexive maxima at $\lambda_{1\text{-max}}$ = 580.49 nm and $\lambda_{2\text{-max}}$ = 694.32 nm and a reflectance minimum is constant localized at $\lambda_{3\text{-min}}$ = 631, 02 nm (fig. 1.c). At this wavelength (λ= 631.02 nm), the absorption of the yeast cultures treated with MB is

maximum and the photodynamic effect on the yeast can be induced by laser radiation with corresponding wavelength.



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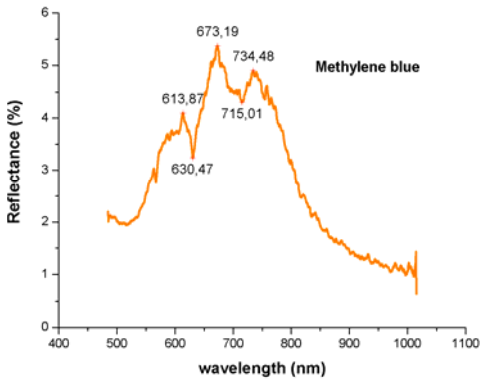

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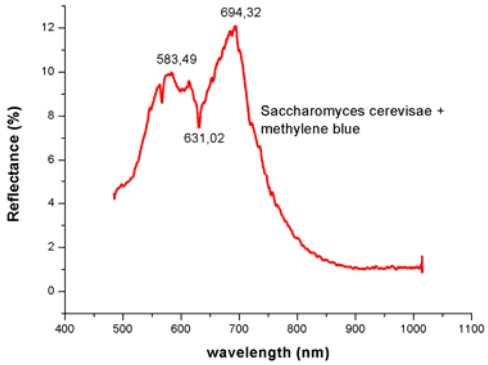

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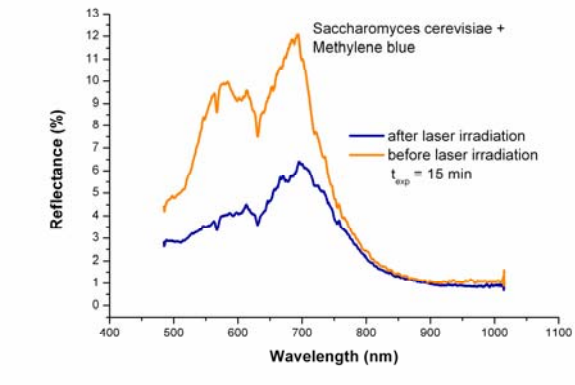
Fig. 3. Diffuse reflectance spectra a) Saccharomyces cerevisae culture; b) methylene blue solution; c) Saccharomyces cerevisiae culture with methylene blue

After exposure of the yeast cultures treated with MB at laser radiation for: 15 min, 20 min and 30 min, the diffuse reflectance has decreased (fig 4)



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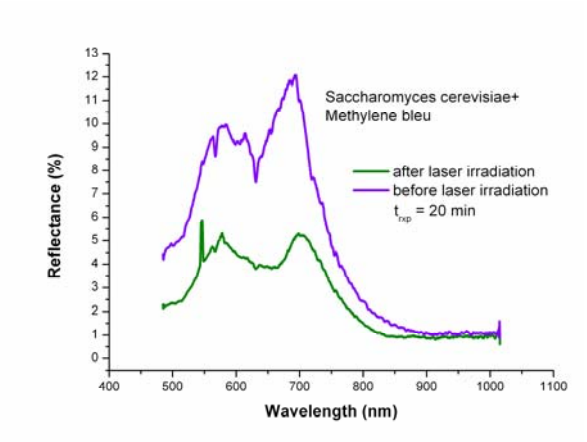

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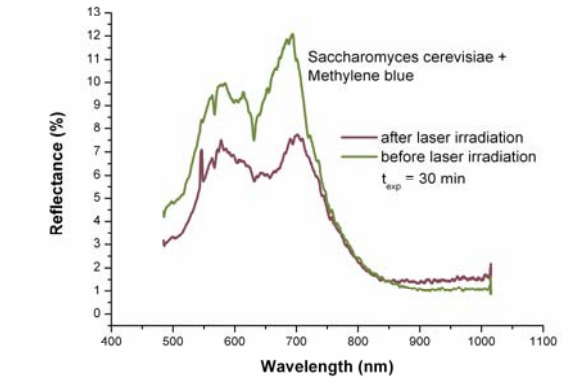

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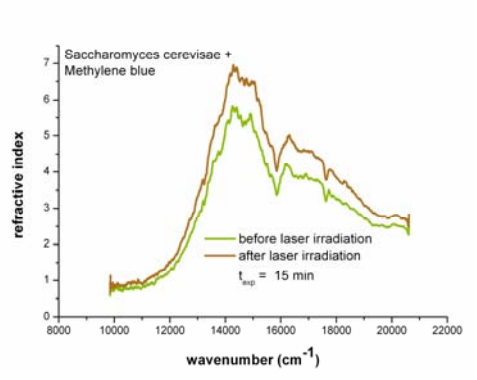
Fig. 4. The variation of Saccharomyces cerevisae cultures diffuse reflectance during photodynamic inactivation a) t_{exp} = 15 min; b) t_{exp} = 20 min c) t_{exp} = 30 min

The photodynamic inactivation of *Saccharomyces* cell is dependent on the irradiation time. Increasing the time exposure to the laser radiation up to a certain value results in the decreasing of diffuse reflectance after laser irradiation and an increasing inactivation rate of the experimental group. The diffuse reflectance of control group increases in time (Table 1).

Table 1. The variation of diffuse reflectance during photodynamic inactivation of yeast cultures.

Exposure time (min)	R_{med} (%)		
	EG		CG
	Before laser irradiation	After laser irradiation	
15	8.33657±0.01601	3.9703±0.01789	6.2801±0.01372
20	8.53023±0.01753	4.10992±0.01505	6.5214±0.01647
30	8.67903±0.01425	5.9417±0.01625	6.7256±0.01498

EG – experimental group
CG – control group
R_{med} (%) – reflectance at λ = 635 nm



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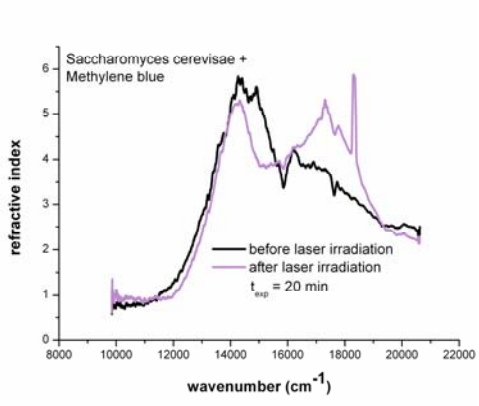

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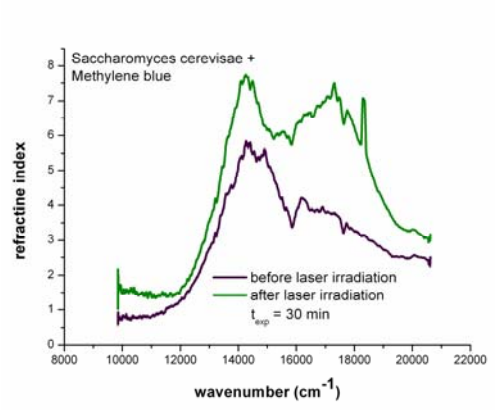

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Fig. 5. The variation of Saccharomyces cerevisae cultures refractive index during photodynamic inactivation.

Table 1 shows that the exposure of the yeast cultures for 20 minutes to laser radiation induces the largest drop of diffuse reflectance due to the photodynamic effect induced by laser radiation at a cellular level in the presence of a photosensitizer.

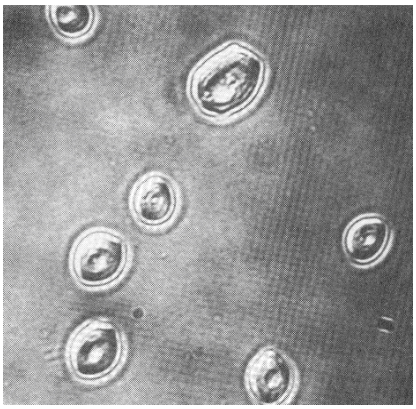
The efficacy of the photodynamic inactivation of *Saccharomyces cerevisiae* cells were evaluated by means of refraction index changes, too, during photodynamic inactivation process.

The Kramers-Kronig analysis has been applied for the determination of the refractive index, and its variation is shown in figure 5. During the laser irradiation, an increase of refractive index has been observed, as a proof for the decreasing of the absorption coefficient of the culture cells.

The refractive index of *Saccharomyces cerevisae* cultures increased vs. exposure time, as it is mentioned in Table 2.

Table 2. Time variation of the Saccharomyces cerevisae cultures refractive index.

t (min)	Refractive index Sccharomyces cerevisiae – MB
15	0,986
20	1,048
30	1,093



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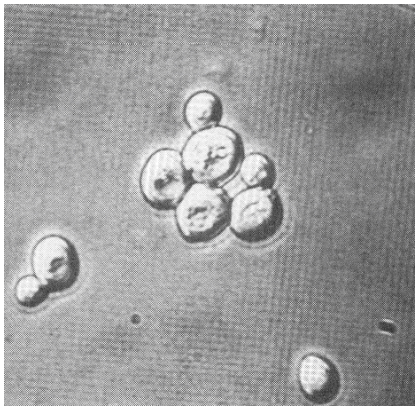

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Fig. 6. The aspects of Saccharomyces cerevisae cells a) before laser irradiation; b) after laser irradiation

The growth of the refractive index shows a low absorbtion of this radiation which is tantamount to a total consumption of the photosensitizer. When the irradiation time is extended with 5 min (from t_{exp} = 15 min to t_{exp} = 20 min), the refractive index grows with 0,188 (n_1-n_2 = 1,048-0,986 = 0,188).

If the exposure time is risen with 10 min (from t_{exp} = 20 min to t_{exp} = 30 min) the variation of the refractive index becomes Δn = 0,045. Thus, by continuing to enlarge the exposure time, the n variation lowers indicating the cessation of some proceses in the culture.

In this case, we can assume that the optimum time of exposure to laser radiation of *Saccharomyces cerevisae* cultures treated with methylene blue as photosensitizer, is 20 min.

This result is also confirmed by the microscopic study made on experimental group exposed to laser radiation for 20 min. (fig 6).

After laser irradiation, an agglomeration and shape for all cells could be an obvious evidence for *Saccharomyces cerevisae* destroying.

4. Conclusion

The efficacy of photodynamic inactivation of the microorganisms depends on a range of factors among which: the concentration and the preparation method of the photosensitizer, the time interval between the administration of the photosensitizer and the exposure of the cells to light radiation, the parameters of the light radiation (wavelength, time exposure, pulse duration, pulse frequency, etc), choosing the methods for the evaluation of the biological response.

In the present study only one of these factors has been analysed for the case of photodynamic inactivation of the *Saccharomyces cerevisae*, this being the time of exposure to laser radiation.

The obtained results have shown that there isn't a linear relation of dependence between the exposure time and the success rate of the treatment. In the studied case, for the *Saccharomyces Cerevisiae* culture cells we could estimate an optimum of exposure time to laser radiation of 20 min, using methylene blue as sensitizer.

In the future all of the other factors mentioned above have to be analysed to determine the optimum treatment conditions so that the fotodynamic inactivation method can be effectively used against different pathogenic and harmful microorganisms.

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