Acta Biologica Szegediensis
http://www2.sci.u-szeged.hu/ABS

ARTICLE

Effects of electric current frequencies, laser irradiation and combined treatment on *Saccharomyces cerevisiae* viability

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ABSTRACT In this research, we examined the influence of low voltage (9 V) electric current frequencies (1.4 and 17 Hz), laser irradiation (648 and 532 nm) and combined treatment (one frequency and one laser beam) on the viability of baker's yeast (*Saccharomyces cerevisiae*). Each treatment was conducted using modified methods and equipment in air-filter equipped working chamber. Staining was performed by a non-vital/vital staining technique that has shown an increase in viability of all samples. Counting of yeast cells in 1 ml of sample gave us several positive results in terms of different treatments, cell viability and increase in the number of healthy cells. Treatment with electric current at higher frequencies (4 and 17 Hz) showed increased cell death counts and, although compensating by an increase in viability, the 17 Hz frequency was considered more hazardous. The most adequate treatments (both increased viability and cell count) were the combined treatments (1 Hz/4 Hz + one of the two laser beams). Although, all electric treatments show certain increases in cell viability, combined treatments (1 or 4 Hz coupled with green or red laser beam) show the most promise in achieving both increased cell viability and increased cell counts.

Acta Biol Szeged 60(2):139-144 (2016)

KEY WORDS

cell viability
electrical current
laser irradiation
Saccharomyces cerevisiae

Introduction

Problems in industrial mass production are concentrated mainly on low success rates in increasing biomass, while also maintaining healthy starting colonies. Due to high demand in this industry sector, there is a need to grow and maintain a high viability yeast culture (especially in large bioreactors with tens of thousands of liters in volume) (Babayan and Bezrukov 1985). Industrial treatments rely mostly on mechanisms discovered through scientific research based on treatment of baker's yeast with electricity or low wavelength electromagnetic radiation. (Karba et al. 1991; Piera V 1992; Yun et al. 1998; Wattanakaroon and Stewart 2000; Teixeira and Mira 2011). These findings point out that yeast cells initially divide swiftly and in great numbers, but then start to recede for unknown reasons as the electric treatment continues (Beschkov and Peeva 1994; Alvarez et al. 2003). According to several authors (Jacob et al. 1981; Blenkinsopp et al. 1992; Martin 1992; Thatipamala et al. 1992; Chi et al. 1995; Fologea et al. 1998; Wang et al. 2005), treating certain types of yeast with electric current leads to lower living and active cell counts. This is due to focus being placed mainly on higher voltages and duration of the treatment itself. Also, it was confirmed that a type of bioelectric effect occurs during treatment, which produces a lower viability ratio in the growth medium (Omori et al. 1996; Stewart et al. 1999; Bairva and Bastila 2010).

The main source of problems for researchers is the asynchronous yeast cell cycle, which increases active yeast cells and creates a negative nutrient feedback, so the desired effects actually become a setback (Rice and Ewell 2001). Problems arise when trying to achieve roughly 10% of healthy cells in the total biomass in larger industrial bioreactors (Peguin and Soucaille 1996). These authors emphasize that treatment with alternating electrical current at 20 V and frequency of 10 MHz may produce acceptable numbers of colony forming units. The issue of numbers of colony forming units persists, when the acceptable number of cells is between 4-9 x 10^8 units/ml, but only in special industrially modified growth mediums (Nakasono et al. 1997; Oppezzo and Pizarro 2001).

By adding experiments with laser irradiation treatment of living cells, we continue to add more effects and possibilities, along with new obstacles, to this work. Laser irradiation treatment schematics can be found in several research experiments (Niemann 1983; Matsunaga et al. 1984; Chen et al. 1998; Petin et al. 2001) where pulsating ultraviolet (UV) and stationary infrared (IR) radiation was used to achieve the phenomenon of photo-inactivation of baker's yeast and other

Submitted October 2, 2016; Accepted December 5, 2016
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microorganisms. These authors indirectly conclude, that a minimum dosage of 68 mJ/cm^2 during a 20 s treatment with a beam pulsating at the source represents a sufficient threshold to excite cells in various ways. Several authors already wrote about the effect of increasing cell viability through different types of irradiation (Blenkinsopp et al. 1992; Piera 1992; Stewart et al. 1999; Wang et al. 2005).

This research combines three different low voltage (9 V) frequencies (1.4 and 17 Hz) of electric current (alternating current - AC), two laser beams (648 and 532 nm, both at 5 mW) and blends these two treatments into a new, combined treatment. Furthermore, by adding the cell viability parameter to cell counts in 1 ml of sample we sought to summarize and visualize changes in treated and non-treated cells, as well as to investigate these methods for potential industrial use.

Materials and Methods

Material

For the purposes of this research we used baker's yeast (special industrial strain of *Saccharomyces cerevisiae*), which we took from a commercial product line (Di-Go© industrial product by Podravka-Croatia) due to its stability and industrial importance.

Prototypes and experimental equipment

The working equipment included several experimental prototype technologies such as:

1.) Modified prototype laser irradiation and combined treatment rectangular receptacles (two were made and each with a total volume of 10 ml; high temperature resistant; equipped with separate red and green Helium-Neon type lasers at wavelengths of 648 and 532 nm; final irradiance of 5 mW during 20 s = 100 mJ/cm^2; lasers were pointing downwards into the test receptacle).

2.) Electric current converter prototype for low voltage AC output with a choice of different frequencies (hand crafted to convert standard 220 V AC to a final output of constant 9 V with a range of selectable frequencies between 1 Hz - 115 kHz). Selected frequencies were conducted via two stainless steel electrodes (45 mm apart) placed on the sides of a purpose-made high temperature resistant receptacle (with a total volume of 360 ml).

3.) Modified working chamber with an air-filter (hand crafted to provide enclosed environment for aseptic type of work; air-filter was coated with several layers of active coal; hermetically sealed during treatments; clean and filtered air pumped inside via air-filter fan; rectangular in shape; total volume of 0.15 m^3).

Preparing the working yeast solution and experimental phase

We prepared a special type of industrial growth medium (working yeast solution - WYS) to act as a base industrial medium identical to those used for growing in bioreactors (Thatipamala et al. 1992). For samples we used a 1% solution of baker's yeast in the industrial growth medium (1.5 g of fresh yeast in 150 ml of industrial growth medium, this process was repeated as many times as needed) with continuous mixing on the magnetic mixing machine (1 min on 300 revolutions). Every experiment had a control series and a treatment series, although combined treatment had an additional control. During this research a total of 840 tests were done (every parameter test was repeated 30 times).

1.) *Low voltage electric current treatment*: 150 ml of WYS was treated with one of the three selected frequencies (1.4 or 17 Hz) for 15 min, while the control sample was placed in the incubation chamber (warm, dry, dark container with a constant temperature of 27 °C). Treated samples were placed next to the control sample during the waiting period (24 h).

2.) *Laser irradiation treatment*: From the WYS we took 10 ml of 1% yeast solution, transferred it to a modified receptacle attached to a laser, and irradiated the sample for 20 s. A control sample (10 ml) was taken and placed together with the treated sample in the incubation chamber for the 24 h waiting period.

3.) *Combined treatment*: For this treatment we applied one low voltage electric current treatment (and took additional control + sample for each of the frequencies) and then irradiated the sample with a laser for 20 s. After the treatments, the double-treated sample and control samples (both regular and additional control) were placed in the incubation chamber for the 24 h waiting period.

Microscopic analysis and gathering the data

We stained the samples with 1% methylene blue stain (Semikem d.o.o. BiH-cat. no. 26071 Metilensko Plavo). Cells were counted using a standard hemocytometer method in the Bürker-Türk counting chamber (DHC-B02 hemocytometer) using a light microscope (Olympus CH20). For photography and detailed analysis we used an advanced light microscope (Olympus BX41) with a mounted digital camera (DP12). For this purpose we used registered official software (Olympus DP12 Soft DP12-CB Ver.01.01.01.42.® Olympus Corp.).

Raw data was expressed as a counted number of cells (living and dead cells differentiated and incorporated into results) and then processed into percentage of viable cells (%), and number of cells in 1 ml of medium (x 10^{10}). Viability was calculated by dividing living cells by dead cells and multiplying by a factor of 100%. The number of cells in 1 ml of solution was calculated by multiplying three factors

Table 1. Cell viability**(in % and 1 ml × 10^{10}) before/after treatment by low voltage electric current and Students *t*-test.

Groups	Control		Treatment		P-values	
	%	$\times 10^{10}$	%	$\times 10^{10}$	%	$\times 10^{10}$
1 Hz	63.31 ± 4.23	4.08 ± 1.80	79.17 ± 5.51	7.74 ± 0.45	0.016*	0.027*
4 Hz	55.50 ± 5,07	3.27 ± 1,29	69.87 ± 6,77	3.83 ± 0,80	0.042*	0.553
17 Hz	54.13 ± 5.08	3.52 ± 2.0	65.97 ± 3.05	3.85 ± 0.54	0.026*	0.795

* signifficance at level 0.05
** all test parameters were repeated 30x

(number of living cells, number of counted quadrants (5) and the dilution factor (10 000)) and dividing the result with the counting chamber volume (0.0001 ml).

Statistical analysis

The data gathered from viable samples was statistically processed using statistical analysis tests such as Student's t-test and single factor ANOVA. Main results were expressed as average values with standard deviations.

Results

Low voltage electric current treatment

According to Table 1, we have the average data (% and × 10^{10}) along with a standard deviation (± SD) for the control and treated group (1.4 and 17 Hz) of samples. Treating the samples with a low voltage electric current of different frequencies caused an increase of viability and overall cell number in all three of the used frequencies. The highest levels of increase in cell viability and cell number, was in the 1 Hz treatment (15.86% increase in viability and 3.66 $\times 10^{10}$ increase in cell number). The other two frequencies (4 and 17 Hz) had a slight increase in cell vi +ability and cell number.

As it could be seen in Figure 1, the differentiation between living and dead cells showed an increase in cells, which were metabolizing the dye at the time of the counting (light blue or dark grey color). These cells were not taken into account as we could not determine if they were dying or actively metabolizing the dye. So only colorless cells were counted as the living and the dark blue/dark grey counted as the dead.

Laser irradiation treatment

Treating the samples with laser irradiation caused an increase in both cell viability and cell number in both experimental groups (Table 2). Although, the highest cell viability increase (5.68%) was in green laser domain (532 nm), the highest cell number increase (3.39×10^{10}) was in red laser domain (648 nm).

Combined treatment

As presented in Table 3, the highest increase in cell viability had treatments of 1 Hz frequency and red laser beam (19.5%) and 17 Hz frequency and red laser beam as well (13.16%), while the lowest increase had the combination of 4 Hz frequency and red laser beam (5.5%). Cell number had highest values in 1 Hz frequency and green beam laser (8.32×10^{10}), 1 Hz and red beam laser (7.7×10^{10}), and 4 Hz frequency and green laser beam (7.45×10^{10}), while the lowest cell number had the 4 Hz frequency and red laser beam (2.2%).

Discussion

Past studies analyzing the effects of low voltage electric current on yeast are not abundant, although fusing of fungal

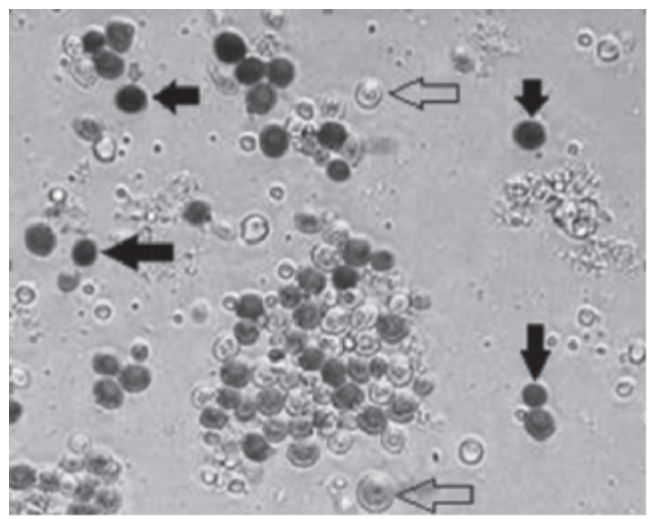

Figure 1. Methylene blue stain (black/white filter) was used to visualize alive (light arrow) and dead cells (dark arrow).

Table 2. Cell viability**(in % and 1 ml × 10^{10}) before/after treatment by laser irradiation and Students *t*- test.

Groups	Control		Treatment		P-values	
	%	×10^{10}	%	×10^{10}	%	×10^{10}
648 nm	67.54 ± 2.84	7.17 ± 0.45	71.82 ± 1.37	10.56 ± 3.26	0.079*	0.148
532 nm	69.49 ± 2.62	7.97 ± 1.29	75.17 ± 0.98	11.02 ± 1.03	0.024*	0.033*

* significance at level 0.05
**All test parameters were repeated 30x

Table 3. Cell viability**(in % and 1 ml × 10^{10}) before/after combined treatment (RL = red laser beam at 648 nm; GL = green laser beam at 532 nm) with single factor ANOVA values.

Groups	Control		Control +		Treatment		P-values	
	%	×10^{10}	%	×10^{10}	%	×10^{10}	%	×10^{10}
1 Hz+RL	55.90 ± 3.75	4.10 ± 0.33	71.50 ± 1.25	9.07 ± 2.0	75.40 ± 0.51	11.80 ± 1.75	0.000*	0.002*
1 Hz+GL	70.08 ± 0.50	7.15 ± 0.92	72.53 ± 1.40	10.87 ± 0.49	76.49 ± 2.70	15.47 ± 1.30	0.012*	0.000*
4 Hz+RL	66.47 ± 2.28	6.72 ± 0.41	70.50 ± 0.65	7.13 ± 0.39	72.97 ± 1.60	8.92 ± 0.38	0.008*	0.001*
4 Hz+GL	66.78 ± 3.34	7.35 ± 0.82	68.12 ± 0.34	8.37 ± 0.33	74.31 ± 0.85	14.80 ± 0.79	0.007*	0.000*
17 Hz+RL	58.90 ± 2.25	7.18 ± 0.38	67.84 ± 3.19	7.53 ± 0.42	72.06 ± 1.44	9.45 ± 0.48	0.003*	0.001*
17 Hz+GL	65.34 ± 1.74	6.43 ± 0.16	67.48 ± 1.34	6.57 ± 0.57	73.32 ± 2.36	8.68 ± 1.88	0.003*	0.003*

* significance at level 0.05
**All test parameters were repeated 30x

cell protoplasts under high voltage pulses was observed in earlier works (Somogyvári et al. 1996). Most of the related studies (Chen et al. 1998; Rodriguez et al. 2003; Schuerger et al. 2006) were focused on the duration of treatment and the voltage. Increasing of the voltage output leads to a decrease in numbers of living cells and in doing so affects actual viability by physically destroying yeast cell walls (if the voltage threshold exceeds 80 V). Extending the duration of treatment can also lead to decreases in viability and cell unit counts, as it prolongs the life cycle of fewer yeast generations. However, several authors (Binninger and Ungvichian 1997; Takeshita et al. 2003) propose the possibility of applicative characteristics of alternative technologies in the industrial sector. During the past few years, low voltage electric current with 1 mA charge was used in research in industrial-level production, however, this creates a higher charge within the bioreactor and can potentially burn down cells. Placing more strain on cells will most likely yield yeast cells with thicker cell walls and prolong cell division, which could produce abnormal cells.

When the samples were treated only with the laser irradiation protocol (either 648 or 532 nm beam) cell viability would rapidly rise for a longer time and would remain at a high induction level, in contrast to the low voltage electric treatment. According to several literature sources (Jacob et al. 1981; Chi et al. 1995; Wellman et al. 1996; Coster and Hillcot 1999; Takeshita et al. 2003), laser irradiation treatment leads yeast cells into a division-ready state as this speeds up their molecular-biological clock. The mechanism of inducing yeast cells to divide (even the cells physiologically not yet able to do) is still not fully understood. According to several authors (Petin et al. 2001; Schuerger et al. 2006; Bairva and Bastila 2010), excitation of specific cyclin-dependent kinases is responsible for activation/control/repression of the cellular life cycle.

According to Somogyvári et al. (1996), electric current pulses (optimally at 200 kHz and at 1 MHz best pearl chain formation) caused better protoplast fusion in *Mucor circinelloides*. This finding supports the fact that high frequencies and high voltage increase the success of fusion of protoplasts, however, this could lyse the walls of other organisms such as baker's yeast.

Based on aforementioned arguments, the results gathered in this research show a far greater increase in cell viability and cell growth under laser irradiation than under low voltage electric current at all three tested frequencies. One of the drawbacks of laser irradiation treatment was the high concentration of energy, which increases cellular temperature and literally fries yeast cells if not carefully calibrated for dosage.

Highly vacuole saturated yeast cells were noticed during treatments with both laser irradiation and electric current, although in much higher percentage in electric current treatments. This type of cells slow down cell growth by competing for more nutrients, while slowly dividing or not dividing at all, thus impairing their cell cycle mechanisms and becoming a colonial burden (Karba et al. 1991; Omori et al. 1996;

Nakasono et al. 1997; Stewart et al. 1999). Treatment series performed with the 532 nm laser indicated an increase in young yeast cells with an average of two newly formed buds on many cells. This situation leads to increased viability rates and a greater number of cell units in samples. For future research we recommend a detailed analysis of buds. Their presence/absence, as well as their size and age, could be a valuable indicative and prediction tool.

Combined treatments with low voltage electric current and either red or green beam laser have shown higher cell viability values and cell unit counts than separate treatment of samples (with only electric current or laser irradiation). Although, high resulting values may be achieved by separate treatments, this increased result is impacted by the fact that these treatments take relatively less time than combined treatments. By combining first the low voltage electric current and then laser irradiation, we actually prepare yeast cells for the shock-plateau, and when we irradiate them we have prolonged their cell division cycle by adding a small amount of irradiation energy.

Aggregation of yeast cells in colonies often leads to increased mortality rates. The presence of competition for nutrients is easily observed by (microscopically) analyzing samples and seeing smaller cells along with abnormally large ones. This competition is present even in nutrient rich mediums. Nutrient competition is also very common in control series and series, which need a 24 h waiting period. The logical assumption would be that this competition is forcing yeast cells to grow unhindered, while at the same time reducing available nutrients and pH values.

The amount of laser irradiation must be experimentally determined prior to treatment, because irradiation may trigger apoptotic cell mechanisms, which destroy cells much faster than the actual treatment. For this reason, combined treatments were found to be the most suitable. In these treatments the samples blasted with laser irradiation must cross over the energy excitation threshold (this threshold is raised by prior treatment with low voltage electricity) to actually affect the cells.

Cell viability results gained after 1 and 4 Hz treatments show a major advance in low-to-none collateral damage treatments. If we add the 17 Hz frequency, we get another improved treatment for increasing cell viability and cell growth, however, its higher frequency nature presents some limitations. The 17 Hz frequency increases cell viability rapidly, but then it becomes static and starts to kill yeast cells at a faster rate.

The 4 Hz frequency has shown to induce the budding process in young yeast cells, which led to increased cell viability (budding intensity is of indicative nature!). Due to its higher pulse nature, this frequency had a positive effect on overall cell number.

By forming new aggregated colonies, many yeast cells start to die out due to over-consumption of nutrients and higher percentage of already dead cells in these colonies. This process leads to subsequent cell viability loss and lower cell unit counts as the dead cells become a burden to the colony. With microscopic analysis of fresh slides treated with 4 Hz frequency, we noticed more budding young cells as well as large storage vacuoles in cells, which was explained by multi-sporulative existence of a haploid nature (even though the cells were diploids there was a possibility of forced polyploidy, which meant a shorter life cycle). In contrast to the 1 Hz, the 4 Hz frequency is more suitable for industrial applications as it provides a sufficient viability increase factor and is cheaper to produce.

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