Biological autoluminescence for assessing oxidative processes in yeast cell cultures

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ABSTRACT

Nowadays, modern medicine is looking for new, more gentle and more efficient diagnostic methods. A pathological state of an organism is often closely connected with increased amount of reactive oxygen species (ROS). They can react with biomolecules and subsequent reactions can lead to very low endogenous light emission (biological autoluminescence - BAL). This phenomenon can be potentially used as a non-invasive and low-operation-cost tool for monitoring oxidative stress during diseases. To contribute to the understanding of the parameters affecting BAL, we analyzed the BAL from yeast *Saccharomyces cerevisiae* as a representative eukaryotic organism, The relationship between the BAL intensity and amount of ROS that originates as a result of the Fenton reaction as well as correlation between spontaneous BAL and selected physical and chemical parameters (pH, oxygen concentration, and cell concentration) during cell growth were established.

Introduction

One of the main missions of modern society is to prevent, manage, and cure diseases. A key requirement to achieve this is the development of effective and ideally non-invasive diagnostic methods. New gentle methods will help to overcome limitations of current methods which can be painful for the patient and destructive for the analyzed biosamples. This mission goes beyond human diseases, as it also extends to diseases of animals or plants, which have a strong impact on agriculture and the food industry. In this work, we contribute to the development of a non-invasive biophotonic method for monitoring oxidative processes in cell cultures, as model organisms. Multiple diseases, including those that are the leading causes of deaths worldwide, such as cancer[1], neurodegenerative[2], and cardiovascular[3,4] diseases, are closely connected with an increased amount of reactive oxygen species (ROS) in affected body parts. In living organisms, incomplete reduction of O_2 during respiration is considered to be a typical source of ROS. However, ROS are also naturally produced during fatty acid metabolism in peroxysomes and by NADPH oxidases in the membrane of macrophages[5]. Non-mitochondrial ROS sources, originating from proteins encoded by NADPH oxidase orthologs, have also been recently discovered in single cell organisms, such as yeast[6]. Therefore, organisms have developed various defense mechanisms to eliminate ROS or the damage caused by them. However, an imbalance between ROS production and the capability of an organism's defense system to eliminate them leads to oxidative stress and harm of an organism.

Interactions of ROS with biomolecules lead to a cascade of reactions. One of the branches leads to the formation of unstable high-energy intermediates such as dioxetanes[7] and tetraoxides[8], which decompose to generate electronically excited species[9]. Redundant energy can be released in the form of heat or as light emission[10]. The probabilities of reactions leading to creation of excited species and subsequent radiative de-excitation are quite low, therefore the intensity of photon emission is also really low[9]. In addition to biological autoluminescence (BAL), other terms such as ultra-weak photon emission, biophotonics, and bio-chemiluminescence are used for this phenomenon[10]. Thus, amount of ROS in a studied sample is closely correlated with the BAL intensity. Although the probable general biochemical and biophysical mechanisms of BAL are rather well established[9], the origin and physiology of the processes leading to BAL are different for different organisms in different condition (e.g.[11–14].

Yeast *Saccharomyces cerevisiae* is a widely used and well characterized model organism in molecular biology research. Furthermore, yeasts and their various strains are also essential in the food industry[15,16] and biotechnology[17]. They are eukaryotic the same as humans, animals or plants but single-cell; thus, it is easier to evaluate effects of various stress factors on the organism. Therefore, we chose yeast *Saccharomyces cerevisiae* as a model organism to analyze ROS-BAL correlations.

Amount of ROS in organisms can be affected naturally as mentioned above but also artificially. Various types of ROS can be generated by adding appropriate reagents into the sample. Such as reaction of Fe^{2+} with hydrogen peroxide (H_2O_2) called the Fenton reaction leads to creation of highly reactive hydroxyl radical besides other products[18].

There are several studies of spontanoues BAL from yeast for example from Quickenden et al.[11,19,19,20]. They studied the intensity and spectral distribution of spontaneous BAL in exponential and stationary phase of yeast growth. Other researchers

used various methods to study oxidation caused by the Fenton reaction[21,22]. Ivanova et al.[23] focused on mechanisms of chemiluminescence as a result of the Fenton reaction. To connect these topics and broaden our knowledge about them, we decided to evaluate correlation between BAL of a living organism, yeast *Saccharomyces cerevisiae*, and artificially increased amount of ROS by the Fenton reaction. We also extended previous measurements of spontaneous BAL of yeast and investigated the correlation of spontaneous BAL with selected physical and chemical parameters.

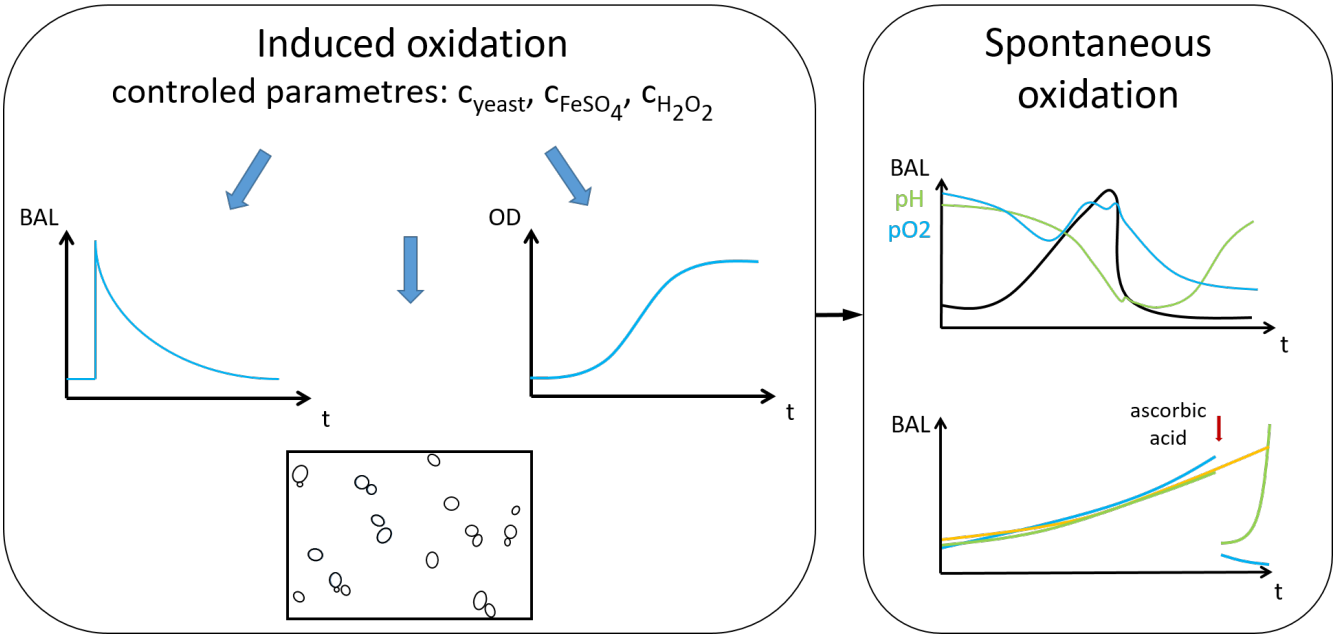

Figure 1. A scheme of the article. Amount of free radicals in a sample was regulated (increased) by the Fenton reaction and it was observed effects on a studied system. It was studied effects of various concentration of yeast and Fenton reagents ($FeSO_4$ and H_2O_2) on biological autoluminescence mainly. Measurement of growth curves and microscopic pictures were also evaluated. It was measured spountanous BAL and its correlation with several selected parametres (pH, pO_2, concentration of antioxidant ascorbic acid), too.

Results and Discussion

Biological autoluminescence enhanced by Fenton reagents

First, we evaluated BAL signal from a yeast sample at 3 different concentrations. The BAL intensity was observed during real-time oxidation of yeast cells with 0.5 mM $FeSO_4$ and 2.5 mM H_2O_2. The kinetics during the first 60 s are shown in Figure 2A. Hydrogen peroxide was injected into the sample after 10 seconds of background measurement. The sums of the BAL signal during the first 60 s after injection of H_2O_2 with a subtracted background (first 10 s before the injection of H_2O_2) are shown in Figure 2B. The average from 3 independent measurements and standard deviation are displayed. We can see that the BAL signal increase together with the yeast concentration up to the limit 10^8 cells·mL^{-1}. Then the sample starts to be too dense and the BAL intensity goes down. Therefore, for subsequent experiments we used a yeast concentration of 10^8 cells·mL^{-1}, that exhibits the highest BAL intensity.

With increasing yeast concentration, the amount of biomass that ROS can react with is higher and the amount of potential sources of BAL grows. However, samples that are too dense sediment quickly, making the lower layers inaccessible for oxygen and the Fenton reagents. The actual amount of biomass that could be oxidised is reduced and the BAL signal also decreases.

We measured the BAL response of yeast samples at concentration 10^8 cells·mL^{-1} induced by the addition of various concentrations of the Fenton reagents $FeSO_4$ and H_2O_2. The final concentration ranges of the reagents were $5{\cdot}10^{-5}$ – 0.5 mM $FeSO_4$ and $25{\cdot}10^{-3}$ – 25 mM H_2O_2. The sums of the BAL intensities during the first 60 s after injection of H_2O_2 with a subtracted background (calculated from the previous 10 s before the injection of H_2O_2) are show in Figure 3. The given numbers (represented by colours) are the average from 3 measurements with a standard deviation. At the lowest used concentrations (0.05 μM $FeSO_4$ and 25 μM H_2O_2), a signal comparable with the intensity of a control sample (only Q water without yeast) was obtained, Supplementary S1. For the measurements at higher concentrations, the BAL signals were significantly higher than those obtained with the control samples. Generally, higher Fenton reagent concentrations resulted in a higher BAL signal. A

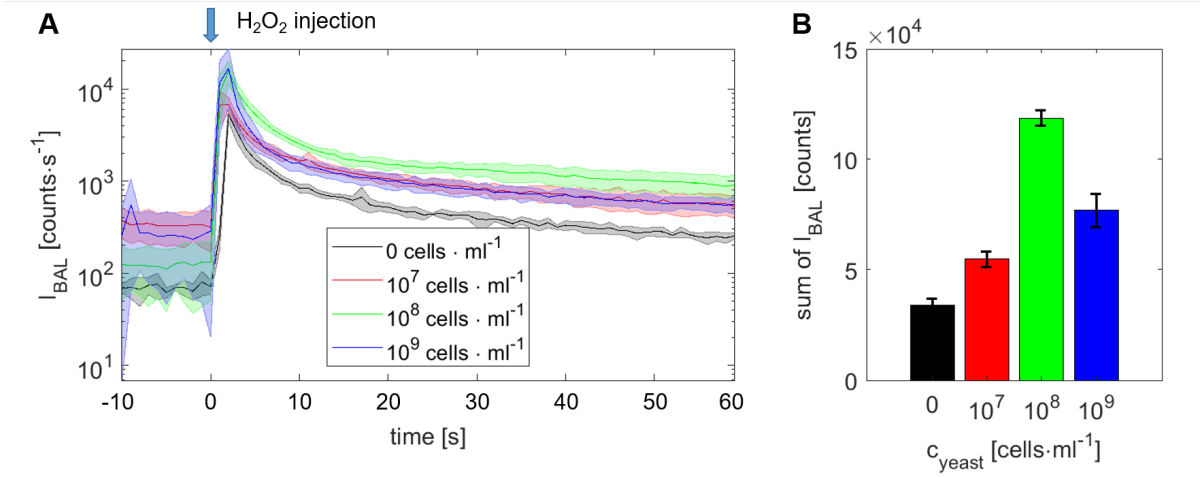

Figure 2. Induced BAL of yeast by the Fenton reaction (0.5 mM $FeSO_4$, 2.5 mM H_2O_2) for different concentrations of yeast (10^7 - 10^9 cells·mL^{-1}). A) BAL kinetics after the injection of hydrogen peroxide into the sample after 10 seconds of background measurement (t = 0). B) Bar graph of the sum of the BAL signal during the first 60 s after injection of H_2O_2 with a subtracted background (first 10 s before the injection of H_2O_2). In both cases, the average from 3 measurements and a standard deviation are shown.

greater difference (at least 100 times) between the concentrations of one reagent resulted in a significant difference between the corresponding BAL intensities, whereas smaller differences in reagent concentrations did not result in a significant difference in BAL signals (such as BAL signal sum for 5 μM $FeSO_4$ and 0.25 or 2.5 mM of H_2O_2).

The influence of 10 min oxidation caused by the Fenton reaction (0.5 mM $FeSO_4$, 2.5 mM H_2O_2) on a yeast sample was evaluated under a microscope (Fig. 4). The yeast concentration (evaluated for cells with 3 – 9 μm in a diameter) was 10^8 cells·mL^{-1} for control sample and before oxidation (Fig. 4, part A) and $3{\cdot}10^7$ cells·mL^{-1} after oxidation of the sample (Fig. 4, part B). We did not observe any damaged cells. However, the yeast cells tended to cluster after oxidation. Cell aggregation could be one of the possible reasons for the lower yeast concentration that was measured in the 3 – 9 μm cell diameter range.

The toxic concentration of hydrogen peroxide is approximately 5 mM in *Saccharomyces cerevisiae* [5, chap. 6, p.246] but inducing the apoptosis depends on the incubation time besides other factors. There are also several factors that can increase the resistance to oxidative stress. Yeast has a higher resistance in the stationary phase and during respiration. Also, previous mild stress condition (including different types of stress) can lead to increased tolerance to oxidative stress. For example, the sudden transfer of yeast cells from room temperature to 30 °C and change of the medium can be experienced as stress [5, chap. 6].

The effect of the Fenton reaction (ca. 10 min; 0.5 mM $FeSO_4$, 2.5 - 250 mM H_2O_2) on yeast growth were also evaluated. As shown in Fig. 5, the samples treated with a higher concentration of hydrogen peroxide had a longer lag phase. However, the final amount of cells (in the stationary phase) was similar for all samples. The duration of the lag phase depends on several factors such as the temperature, aeration, the conditions in a previous environment, amount of transferred biomass, or yeast health [24, chap. 9]. More stressed cells probably need more time to overcome unfavourable consequences and prepare themselves for dividing.

Spontaneous BAL

We also analyzed the spontaneous BAL of yeast culture in a bioreactor during their growth (Fig. 6). Because the sample was prepared under normal laboratory light conditions, we observed an initial gentle decrease of the BAL signal when the sample adapted to the dark condition in a black box. Than, the signal increased during several hours. At the same time, the pH decreased and pO_2 displayed nonlinear behavior. After ca. 12 hours of measurement, the BAL signal sharply decreased, whereas the pH and pO_2 slightly increased. Quickenden et al. observed that the BAL of yeast *Saccharomyces cerevisiae* was similar to nutrient luminescence in anaerobic conditions (nitrogen instead of air)[20]. However, there was still enough oxygen in our samples after ca. 12 hours 6. As shown in Fig. 5, a change from exponential to stationary phase on the growth curve occurs after approximately 12 hours without H_2O_2 treatment. This change of phases is called the diauxic shift and it is connected with a change in yeast metabolism from fermentation to aerobic respiration. Therefore, a sharp decrease of the BAL intensity could be caused by inner changes related with the initiation of respiration.

Because incomplete reduction of O_2 during respiration is usually considered as the major source of ROS[5], organisms probably activate defense mechanisms, which leads to a decreased amount of ROS in cells or repairing of the damage caused by

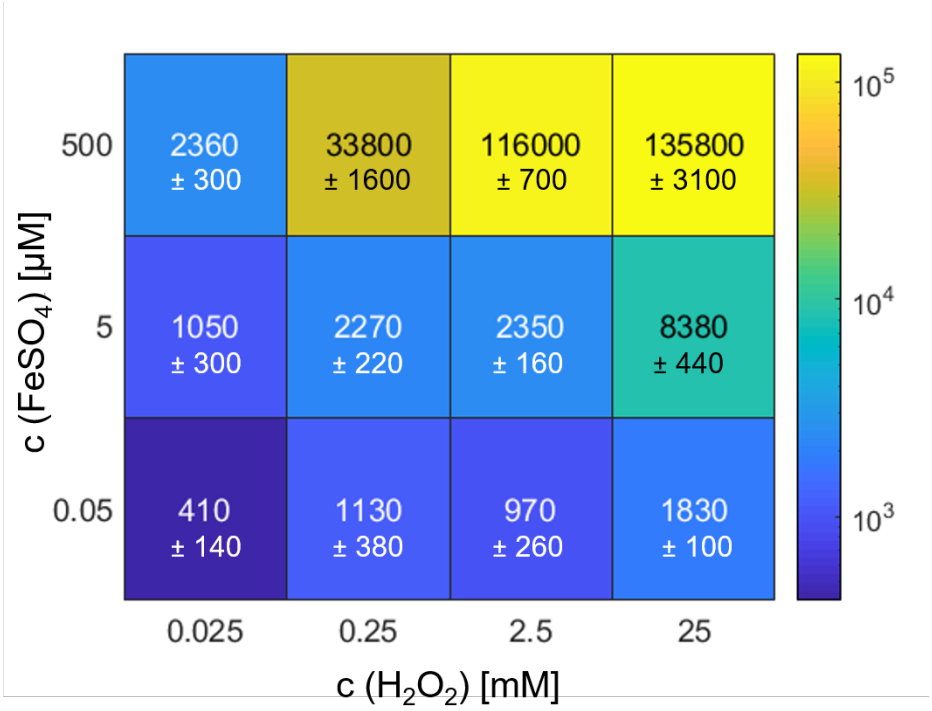

Figure 3. Induced yeast BAL by the Fenton reaction for various concentrations of Fenton reagents ($5{\cdot}10^{-5}$ – $5{\cdot}10^{-1}$ mM $FeSO_4$, $25{\cdot}10^{-3}$ – 25 mM H_2O_2) and a fixed yeast concentration (10^8 cells·mL^{-1}). The colour indicates the sum of the BAL intensities during the first 60 s after injection of H_2O_2 with a subtracted background (the previous 10 s before the injection of H_2O_2). The given numbers are the average from 3 measurements and the standard deviation.

them. Maslanka et al. observed a dependence of ROS amount and also ROS source on the type of carbon source/concentration of glucose and on the type of metabolism[25]. Their results showed that non-mitochondrial ROS sources are an important pool of ROS in yeast, especially during fermentation. They also observed that more ROS are generated by yeast in the medium with higher concentration of glucose when they estimated the level of ROS with dihydroethidium (DHET). These results are in compliance with our data: after the BAL signal reaches the peak and drops to a low level, the addition of glucose causes an increase in BAL intensity (Fig. S3).

According to[25], non-mitochondrial ROS generation can be related to cellular biosynthesis. Tilbury et al. also suggested that possible source of BAL during the exponential phase of yeast growth (during fermentation) are oxidative side reactions accompanying protein synthesis[11]. Rinnerthaler et al. found that important non-mitochondrial source of ROS in yeast is a NADPH-oxidase ortholog Yno1p/Aim14p[6]. Maslanka et al. also evaluated the content of pyridine nucleotide cofactors and the ratios of their reduced and oxidised form. They observed a higher ratio of NADPH/NADP$^+$ for yeast cultivated in the media supporting aerobic respiration[25]. Besides other functions, NADPH is considered to directly operate as an antioxidant to scavenge free radicals and reduce oxidised biomolecules[?]. Jamieson et al.[26] observe significantly higher the (oxidative) stress resistance during respiratory growth and stationary phase than during fermentation. Their results support our suggestion about more effective detoxification during respiration.

Upon a decrease of the pH of the sample during fermentation, the excretion of organic acids and absorption of basic amino acids make a significant contribution[27].

The amount of ROS was usually regulated or rather increased by the Fenton reaction. From greater volume of sample it was also possible to observe the influence of the antioxidant ascorbic acid that decreasing ROS in a studied system (Fig. 7. The effect of the antioxidant depended on its final concentration in the sample. After injection of ascorbic acid at a lower concentration (0.1 mM), we observed a rapid decrease but also a steep increase of the BAL intensity to the values expected for a sample without antioxidant treatment. The injection of ascorbic acid at a higher final concentration (1 mM) resulted in a steep decrease and no renewal of the signal. The observed effects probably depended on the ratio of the amounts of ROS and antioxidant. At a lower final concentrations of antioxidant, the molecules of ascorbic acid react with ROS and are quickly depleted. Then amount of ROS, and thus the BAL signal, increases again without any hindrance. A higher concentration of ascorbic acid either terminates the avalanche production of ROS or considerably reduces the amount of ROS for such a long

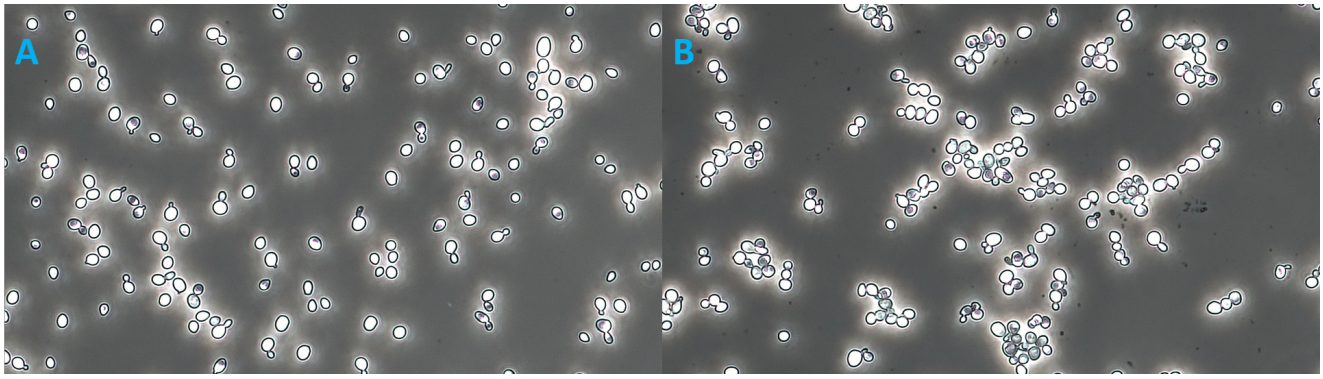

Figure 4. Microscopic pictures of yeast sample in water before (part A) and after (part B) the Fenton reaction (∼10 min; 0.5 mM $FeSO_4$, 2.5 mM H_2O_2). Yeast concentrations (3 – 9 μm in a diameter) were 10^8 cells·mL^{-1} before and $3{\cdot}10^7$ cells·mL^{-1} after oxidation.

time that the yeast can pass through fermentation to aerobic respiration. Červinková et al.[28] also studied the effects of ascorbic acid (vitamin C), among other antioxidants, on yeast BAL intensity. They tested concentrations of 1, 5 and 10 mM and also did not observe any renewal of the BAL signal. They also tested the influence of ascorbic acid (1, 500 and 1000 μM) on HL-60 cells. Even at the lowest used concentration (1 μM), the signal didn't renew. It appears that each sample has different threshold antioxidant concentration and also a different response to various antioxidants[28].

Conclusion

A higher concentration of yeast in the sample resulted in a higher BAL intensity up to the limit (10^8 cells·mL^{-1}). If the sample is too dense, fast sedimentation occurs, resulting in worse accessibility to a large part of the sample for the oxygen and Fenton reagents. Therefore, the BAL signal decreases if the yeast concentrations is higher than the limit. The induction of stress through the addition of higher concentrations of Fenton reagents resulted in higher BAL intensities. At the lowest used concentrations (0.05 μM $FeSO_4$, 25 μM H_2O_2), which are comparable with physiological concentrations in a body, a signal strength comparable with the BAL intensity of the medium alone was observed. Higher concentrations of Fenton reagents induce BAL signals that are significantly above those of the medium background.

After ca. 10 min oxidation of a yeast sample with 0.5 mM $FeSO_4$ and 2.5 mM H_2O_2, we did not observe any damaged cells under a microscope, just the tendency of the cells to cluster. On the other hand, a higher H_2O_2 concentration (25 and 250 mM) caused prolongation of the lag phase of the yeast growth. However, the total cell amount (in the stationary phase) stayed same for all treated samples. Cell wholeness after oxidation can be caused by relatively not so high concentration of Fenton reagents and quite short incubation time or by higher cell resistance to oxidative stress resulting from various causes. It seems that more stressed yeast cells need more time to recover after strong oxidation and to launch reproduction processes.

We also observed progressive increase of the BAL signal from growing yeast culture in a bioreactor up to approximately the twelfth hour, after which the BAL intensity sharply falls and remains at a quite low level. At the same time, the pH decreases, slightly increases at approximately the twelfth hour, and than continues to decrease. The change in pO_2 was nonlinear; however, a small local maximum was evident at approximately the twelfth hour. The yeast growth curve of the non-treated sample shows a change of phases from exponential to stationary (diauxic shift) also after approximately 12 hours of yeast growth. Therefore, the steep decrease of the BAL signal could be probably connected with the change of yeast metabolism (from fermentation to aerobic respiration). On the other hand, active mitochondria are considered as one of the major ROS sources. So it seems that non-mitochondrial sources of ROS also play an important role in their production and that cells also activate an effective defense system against ROS during the change of metabolism to respiration. The sharp decrease of BAL signal from growing yeast culture was also observed after decreasing amount of ROS by the antioxidant ascorbic acid. The BAL signal remains low after injection of ascorbic acid at the higher final concentration (1 mM). However, lower concentration of the antioxidant (0.1 mM) is probably depleted quickly and BAL signal recovers within one hour.

Methods

Yeast *Saccharomyces cerevisiae* wild type (BY 4741) was used as a model organism. They were cultivated for approximately 24 hours in an orbital shaking incubator (Yihder Co., Ltd; LM-420D) at 30 °C, 180 rpm, in standard YPD medium (1 % yeast extract, 2 % peptone, 2 % D-glucose). Then, they were centrifuged (Haraeus Biofuge Stratos) 2 times and the YPD medium

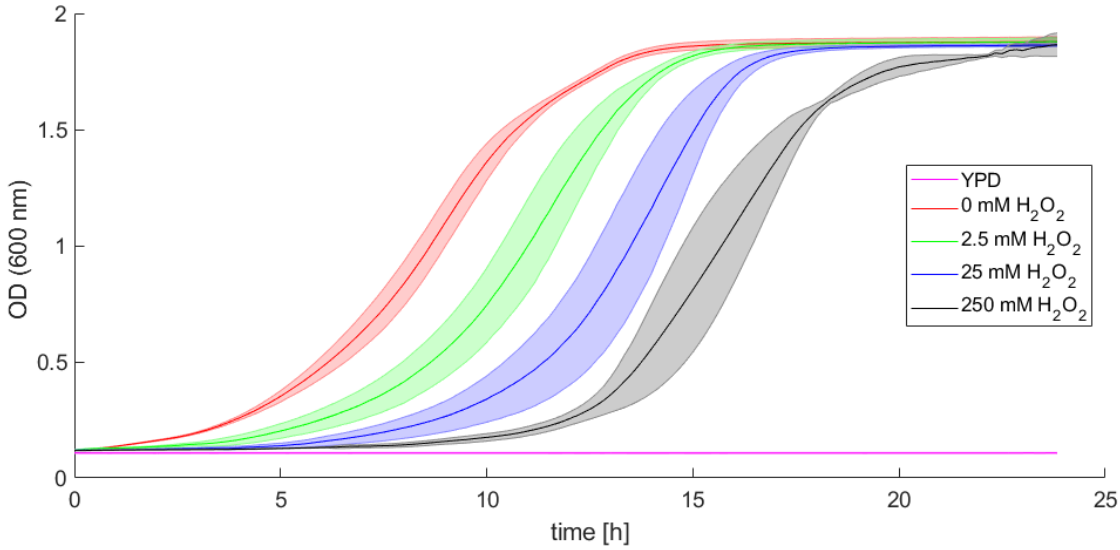

Figure 5. Yeast growth curves after oxidation of samples by the Fenton reaction. Yeast samples (concentration 10^8 cells·mL^{-1}) were oxidised in water for ∼10 min (0.5 mM $FeSO_4$, 0 / 2.5 / 25 / 250 mM H_2O_2). Then the samples were centrifuged and water was changed to YPD medium. Samples were diluted 100 times and cultivated in a Spark microplate reader at 30 °C for 24 h. Measurement of OD at 600 nm were performed every 10 min and the samples were shaken for the rest of the time.

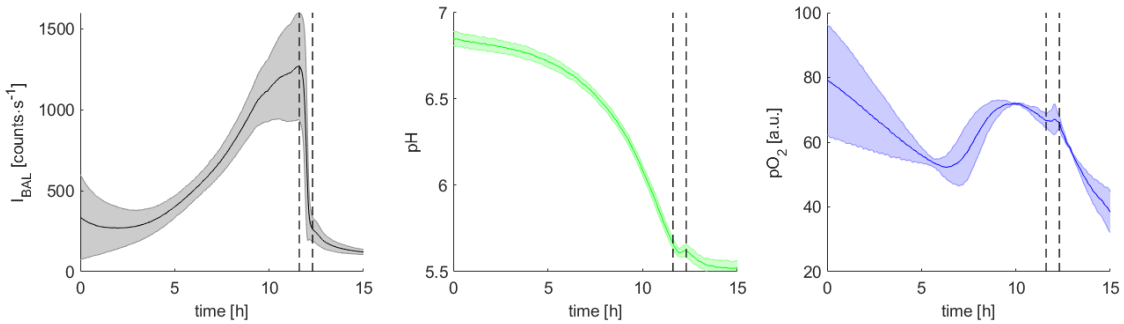

Figure 6. Spontaneous BAL of yeast measured in a bioreactor together with the change in pH and pO_2 (average from 3 measurements with a standard deviation).

was changed to Q H_2O. Measurement of yeast concentration was performed with a cell counter (Beckman Coulter, Z2 series) in the range 3 - 9 μm.

Biological autoluminescence measurement setup

Induced BAL

Induced emission was measured from a 3 mL of yeast sample in Milli-Q water in a Petri dish (Thermo Scientific, diameter 35 mm). The concentration of yeast was between 10^7 and 10^9 cells·mL^{-1}. The amount of ROS in the sample was regulated by the Fenton reaction

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\cdot} + OH^- \tag{1}$$

After preparation of a yeast sample at a required cell concentration, 50 μL of iron(II) sulfate was added to the sample so that the final $FeSO_4$ concentrations in the sample was 50 nM, 5 μM, or 0.5 mM. Then the sample was placed into a special black box with a photomultiplier module H7360-01 (Hamamatsu Photonics K.K., spectral range 300 – 650 nm) where the measurements of BAL were performed. After approximately 1 min of BAL measurement, 50 μL of H_2O_2 (final concentrations: 25 μM, 250 μM, 2.5 mM, and 25 mM) was injected into the yeast sample with $FeSO_4$. Whole BAL measurement take approximately 10 minutes. A scheme of the experimental setup is shown in Fig. 8.

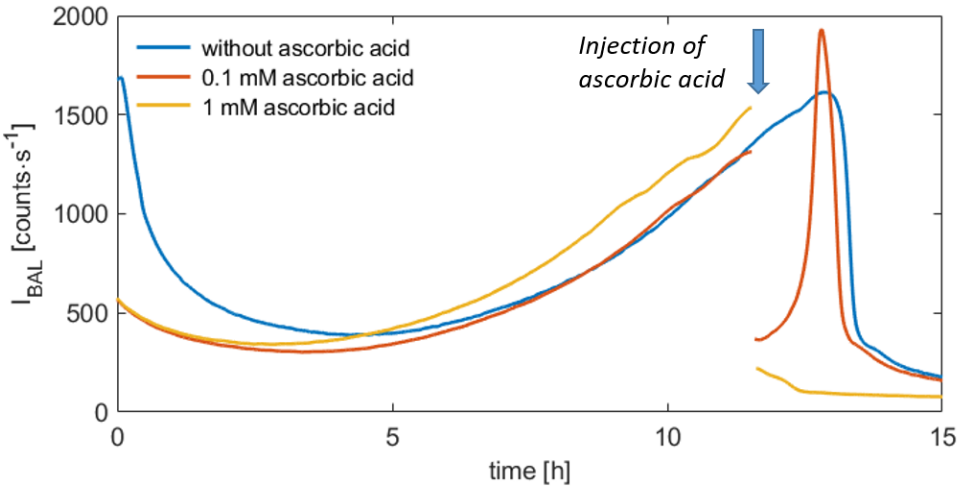

Figure 7. Influence of various concentrations of antioxidant ascorbic acid (0 - 1 mM) on spontaneous BAL of yeast. The initial concentration of the sample was $5{\cdot}10^5$ cells·mL^{-1}.

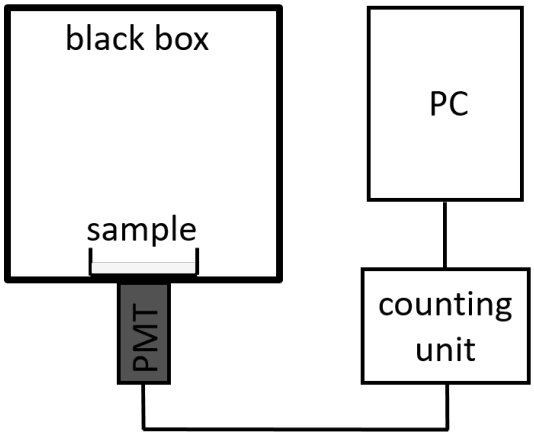

Figure 8. A scheme of the experimental setup for the measurements of BAL during induced oxidation in a Petri dish in a black box

Spontaneous BAL

Spontaneous emission was measured in a bioreactor in a dark room. Yeasts at an initial concentration of $5{\cdot}10^5$ cells·mL^{-1} were stirred, bubbled with air ($\sim$1 l·min^{-1}) and maintain at a temperature of 30 °C during their cultivation in YPD medium. A small amount of antifoaming agent (polypropylene glycol) was added to the 750 mL sample. The measurement time was between 15 and 20 hours. The photomultiplier module (H7360-01, Hamamatsu Photonics K.K., spectral range 300 – 650 nm) was also used for BAL measurements. A scheme of the experimental setup is shown in Fig. 9. Measurement of the pH, pO_2, and temperature was performed by original probes included in the bioreactor.

Growth curve

Growth curves were measured in a Spark microplate reader (Tecan). After approximately 24-hour cultivation of yeast in an orbital shaking incubator (30 °C, 180 rpm) in standard YPD medium, the samples ware centrifuged (2x) and the YPD medium was changed to Q H_2O. Then, 10 min oxidation of the yeast sample (concentration of 10^8 cells·mL^{-1}) was performed with the Fenton reaction (0.5 mM $FeSO_4$, 2.5 mM / 25 mM / 250 mM H_2O_2). The samples were in the shaker (30 °C, 180 rpm) during the oxidation. Then the samples were centrifuged and water was changed to fresh YPD medium. After dilution of each sample 100 times, 200 μL of each sample was put in a well of a 96-well plate (ThermoFisher . . .) and covered with a lid. Each sample was put in the plate 5 - 7 times. The optical density at 600 nm was measured every 10 min for 24 hours. The samples were shaken for the rest of the time. The temperature during measurement of the growth curves was maintained at 30 °C.

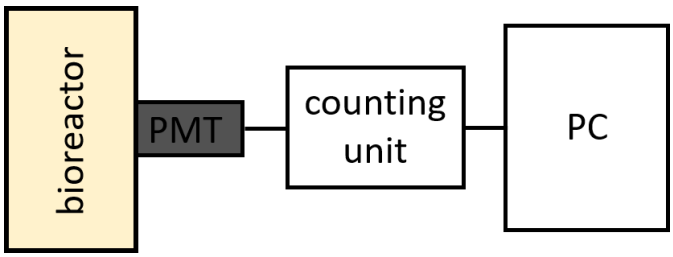

Figure 9. A scheme of the experimental setup for the measurement of BAL during spontaneous oxidation in a bioreactor

Microscopic imaging

Microscopic imaging before and after the oxidation by the Fenton reaction was performed with the microscope BX50 (Olympus) with the camera Moticam 1080. Phase contrast and an objective magnification of 20x was used.

Supplementary Information

Supplementary Information 1

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Conflict of interest

The authors declare that they have no competing interests.

Data availability statement

Data are available on request.

Author contributions statement

Contribution roles according to CRediT:
https://casrai.org/credit/:
P.V.: Data curation (lead), Formal analysis (lead), Investigation (lead), Methodology (equal), Software (equal), Visualization (lead), Writing – original draft (lead)
K.C.: Data curation (supporting), Formal analysis (supporting), Investigation (supporting), Methodology (equal),
M.C.: Conceptualization, Formal analysis (supporting), Funding acquisition, Investigation (supporting), Project administration, Resources, Supervision, Validation, Visualization (supporting), Writing – original draft (supporting), Writing – review & editing (

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