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BIOLUMINOMETRIC ASSAY OF CELL ATP DETECTION UNDER STRESS CONDITIONS AND IN THE ASSESSMENT OF CLEANNESS OF THE FOOD PRODUCING COMPANY

Miroslava Požgajová[1], *Hana Ďúranová*[1]

Address(es): Dr. Ing. Miroslava Požgajová,
[1]AgroBioTech Research Center of the Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, phone number: +421 37 641 4919.

*Corresponding author: miroslava.pozgajova@uniag.sk

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ABSTRACT

Detection and tracking of microorganism's behavior in natural environments and under stressed conditions have been nowadays accelerated by the requirement for the risk assessment associated with environmental contamination. Luminescence-based techniques, for they cost efficiency, fastness, reliability and reproducibility are particularly appropriate for such studies. We used bioluminescence sensing assay to determine ATP levels in the yeast *Schizosaccharomyce pombe*. Determination of adenosine concentration per cell was assessed in the yeast cells counted prior to the experiment. To test the methodology, model organism was exposed to life-threatening concentration of heavy metals cadmium and nickel for three hours. Afterwards, changes in the ATP levels were detected and compared to the control. Strikingly, our results revealed that such conditions lead to considerable increase in the production of ATP by mitochondria compared to untreated cells. Normal level of ATP in the control increased from the basal 9.92×10^{-16} M to 2.90×10^{-15} M after Cd exposure and to 7.58×10^{-15} M upon Ni treatment. The methodology was subsequently used for assessment of cleanness of margarine producing company devices after regular sanitation procedure. Detection of only negligible amount of the ATP signal confirmed good cleaning strategy in the tested food processing company.

Keywords: bioluminescence, ATP, *Schizosaccharomyces pombe*, heavy metal, food processing company

INTRODUCTION

Foodborne pathogens are very assorted in nature and keep causing major public health problems in different countries of the world. Thus, the detection and enumeration of microorganisms either in foods or on food contact surfaces are important tools of quality control or food safety plans. Conventional methods of pathogen detection largely rely on microbiological and biochemical analyses that are highly accurate but time-consuming, and cost-ineffective (**Elegado** *et al.*, **2016**). Due to microorganism growth in culture media followed by other procedures (such as isolation, biochemical and/or serological identification, and in some cases, subspecific characterization), traditional microbiological methods in food industry may take at least 2-3 days to provide initial results (**Bown** *et al.*, **2002**), and up to more than one week for confirming the specific pathogenic microorganisms (**Zhao** *et al.*, **2014**). Moreover, conventional methods may be limited by their elaborateness (**Betts & Blackburn**, **2009**), low sensitivity **(Lee** *et al.*, **2014**) and the risk of false negative results for viable non-culturable pathogens (VBNC pathogens) **(Jen & Chen**, **2017**). In order to prevent spread of infectious diseases, to ensure food safety, leading to public health protection, extensive research has been provided over the last years to invent more rapid and automated approaches of foodborne pathogen detection **(Fung** *et al*., **1988**; **Feng**, **2007**; **Betts & Blackburn**, **2009**; **Jasson** *et al.*, **2010**; **Law** *et al.*, **2015**; **Zhao** *et al.*, **2014**; **Valderrama** *et al.*, **2016**; **Wang & Salazar**, **2016**; **Jan** *et al*., **2018**).
According to the summarized data by **Lazcka** *et al.* (**2007**), rapid detection methods based on biosensors are the fastest growing technologies for pathogen detection compared to PCR, immunology, and gel electrophoresis. Recent advances in bio-analytical sensors have led to the utilization of the ability of certain enzymes to emit photons as a byproduct of their reaction. This phenomenon, known as bioluminescence, is used for cell contamination detection (**Mandal** *et al.*, **2011**). The method is supposed to be the technique giving results in the shortest time (**Dostálek & Brányik**, **2005**).

$$\text{D-Luciferin} + \text{ATP} + O_2 \xrightarrow[Mg^{2+}]{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + PPi + CO_2 + \text{light emission}$$

The adenosine triphosphate (ATP) bioluminescence-sensing assay is considered as an extremely effective biosensor; hence ATP is the energy currency of all living microbes and can be used as a rapid indicator of microbial viability (**Eed** *et al.*, **2016**). During the assay, ATP reacts with luciferin (extracted from the firefly *Photinus pyralis*) in the presence of the catalyst – luciferase enzyme, and the effect of this ATP-dependent oxidation of the substrate is production of oxyluciferin, carbon dioxide, AMP, inorganic phosphate, and emission of light, recorded by a luminometer (**Dostálek & Brányik**, **2005**; **Sygula-Cholewińska** *et al.*, **2014**):
Initially, the oxyluciferin is formed in an electronically excited state, and a light quantum is emitted during the transfer of the product to its ground state (**Lomakina** *et al.*, **2015**). The amount of light produced is proportional to the concentration of ATP, and corresponds to the number of microorganisms in the original sample (**López-Campos** *et al.*, **2012**). Commercially available manual or automated luminometers can detect less than 0.1 pM of ATP per cuvette, corresponding to approximately 100 bacterial cells (**Dostálek & Brányik**, **2005**). Thus, bioluminescence as a rapid method for ATP production can also be employed in cell cytotoxicity determination since ATP serves as the essential immediate donor of energy and is present in all metabolically active cells (**Mandal** *et al.*, **2011**). Regarding this feature, investigation of ATP production by the yeast *Schizosaccharomyces pombe* (*S. pombe*) used as model system provides precise outputs representing moles of ATP produced by a single cell. Furthermore, exposure of *S. pombe* cells to heavy metals (nickel, Ni; cadmium, Cd) has also been included in our study. The fission yeast *S. pombe*, is often used as an unicellular model system for studies of various biological and physiological processes in eukaryotic cells (**Yanagida**, **2002**) mainly due to its unpretentiousness in cultivation conditions, amenability to genetic manipulations, and regular cell size (**Ďúranová** *et al.*, **2019**). As the microbial evaluation of industrial surfaces and devices is useful for monitoring the effectiveness of cleaning and disinfection practices, we applied bioluminescence to assume potential microbiological contamination of devices in a food-producing company after regular sanitation process. In general, luminometric methods serve as convenient approach to measure biological contamination, to its greatest advantages belong relative effortless performance, high sensitivity due to low background, wide dynamic range, and cost effectiveness (**Branchini &**

Southworth, **2017**). Moreover, the promptness in results acquisition (**Cunningham** *et al*., **2011**), allows rapid re-washing of contaminated surfaces or equipment in food producing companies, before production again begins (**Betts & Blackburn**, **2009**).
Current study was thus aimed to determine the ATP mass of defined *S. pombe* cell quantity and its changes after exposure to life-threatening concentrations of Cd (1mM) and Ni (1mM) to validate the methodology. The method was subsequently applied for demonstration of the cleanness of the margarine producing company devices after sanitary treatment.

MATERIAL AND METHODS

Cultures of yeast cells

Schizosaccharomyces pombe (*S. pombe*) was used in our study as a source for ATP production. Culture of the prototroph *S. pombe* strain (JG15458, kindly provided from Dr. Gregan) was grown in standard YE + 5S (yeast extract + supplements) (**Pozgajova** *et al*., **2019**) medium overnight at 30°C and 150 rpm shaking conditions. On the basis of protein fingerprint, the yeast culture was approved as a pure *S. pombe* culture by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Bruker Daltonics, Bremen, Germany). Briefly, cells from the 1mL fresh overnight culture were used for the ethanol-formic acid extraction, covered with 2 μL of matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile with 2.5% trifluoroacetic acid; Bruker Daltonics) subsequently dried on air for 15 min. Protein spectrum of the sample was generated by the Biotyper software, version 2.0 (Bruker Daltonics) and analyzed (**Duracka** *et al.*, **2018**).
Exponentially growing cells were exposed to high concentration (1mM) of nickel (Ni) and cadmium (Cd) for 3 hours. Prior to ATP determination, cells were with the use of Neubauer chamber (VWR International GmbH Graumanngasse 7, 1150 Wien, Austria) counted to evaluate mitochondrial activity expressed as ATP production per cell. To determine ATP levels in single-celled eukaryotic organism $5x10^6$ cells/well were placed to each well of the 96 well plate in triplicate. RLUs (relative light units) of tested samples were compared to RLU of standardized ATP curves and moles of ATP per sample were calculated.

Swabbing protocol of sample collection of the margarine producing company devices

After standard sanitary cleaning procedure using chloride water, detergent, and steam for sterilization a cleanness of the system devices of the margarine producing company was analyzed. Sterile cotton hygiene swabs with metallic applicator sticks (VWR transport swabs, sterile, Copan Italia S.p.A., Brescia) saturated in ATP free water were used to swab controlled equipment covering an area of 10 cm^2. The swab was rotated constantly during swabbing, and each surface was swabbed in two directions ensuring the swab to come in contact with the entire surface area. The cotton applicator was afterwards placed to amies medium containing sterile plastic transport tubes. Sealed tubes and swabs were held on ice until further procedure in the laboratory. The swab was removed from the tube and placed in a sterile eppendorf tube containing 0.3mL of ATP-free water. After thorough mixing excess water was squeezed from the swab by pressing it against the tube wall, and the swab was discarded. To white 96 well plates (Thermo Fisher Scientific, Nunc, Denmark) 100μL of each sample were used for ATP detection in triplicate.

Detection of ATP

Analyses were performed in 96 well plates (white plates) on Glomax Multi+ Combined Spectro-Fluoro Luminometer (Promega Corporation, Madison, WI, USA) with the use of Enliten® ATP assay system (Promega Corporation, Madison, WI, USA) commercial kit aimed to detect 10^{-11} – 10^{-15} M ATP/sample. As the Glomax Multi+ Combined Spectro-Fluoro Luminometer (Promega Corporation, Madison, WI, USA) is equipped with micro-injectors, prior to measurement, 100μL of the Luciferase/Luciferin (rL/L) reagent was injected to each well containing 100μL of the sample. Results are represented as RLU and ATP content is calculated according to ATP standard curves generated with the use of ATP standards provided by the manufacturer diluted in ATP free water and ranging from concentrations 8 to 0.5nM ATP. ATP-free water was used as negative control, RLU values equal or lower then RLU values of ATP-free water were assessed as negative (meaning, no contamination detected).

Statistics

Four replicates were performed for the analysis in each treatment. To determine significance of any acquired differences a Student's *t* test was used. The limit of statistical significance was set up at $P < 0.05$(*), 0.01(**), 0.001(***) for all statistical analyses.

RESULTS AND DISCUSSION

Microbial ATP production

It has been previously reported, that rapid detection of microbial presence even in very low concentrations is possible via detection of ATP levels with the use of bioluminescence technique (Squirrell *et al.* 2002). Relation between ATP concentration and determined RLU values was expressed through correlation standard curve with calibration formula RLU=1011.8ATP + 82.99 (r_P=0.992; P<0.001). Determination of ATP concentration of known cell counts in our laboratory conditions is summarized in Table 1.

Table 1 ATP production in yeast *Schizosaccharomyces pombe*

***S. pombe* per mL**	**cells per well**	**ATP [nM]**	**ATP [M] per cell**
$61.5x10^6$	$5x10^6$	4.96±0.07	$9.92x10^{-16}$

To further test the sensitivity of the used ATP detection method, we exposed the model organism to high concentrations of two different heavy metals, cadmium and nickel, known to interfere with the intracellular homeostatic system.

ATP production under stress conditions

Cadmium and nickel are heavy metals which have received a great deal of attention not only as potent hazards to human health but also for their non-degradable nature leading to persistence in the environment and bioaccumulation via the food chain (**Amari** *et al.*, **2017**). The effect of Cd and Ni on ion homeostasis in *S. pombe* have been assessed in our recent study by **Pozgajova** *et al.* (**2019**). The authors have found that high concentration of applied heavy metals resulted in considerable elevation of Ca^{2+}, Na^{+}, Mg^{2+}, Cu^{2+}, Fe^{3+} levels and significantly decreased content of K^{+} in the yeast cell. However, ATP production measured by luminescence in the yeast cell exposed to Cd and Ni have not been analyzed prior to our experiments.
Using ATP bioluminescence-sensing assay we have found that addition of 1mM Cd or 1mM Ni to the growth media of *Schizosaccharomyces pombe* resulted in marked increase of ATP levels in the cells (Table 2).
Table 2 Moles of ATP per cell treated with heavy metals compared to untreated control

S. pombe	**control**	**Cd [1mM]**	**Ni [1mM]**
ATP [M] per cell	$9.92x10^{-16}$	$2.90x10^{-15}$	$7.58x10^{-15}$

Furthermore, compared to the control group (4.96±0.07nM ATP per sample), the cells exposed to Cd generated 2.93 times more ATP (14.52±0.46nM ATP), and those treated with Ni exhibited even more dramatic increase of the luminescence signal indicating 7.64 times more ATP levels (37.90±2.96nM ATP; Figure 1).

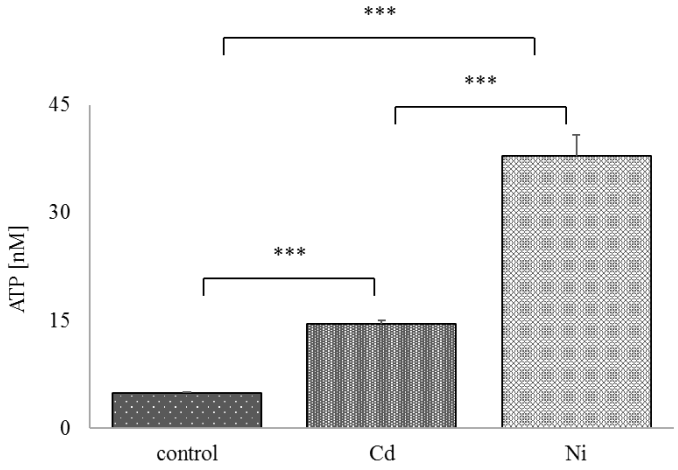

Figure 1 Heavy metal treatment causes marked elevation of ATP levels
Comparison and graphical expression of ATP levels [nM] of untreated and heavy metal treated cells. The limit of statistical significance was set up at $P < 0.05$(*), 0.01(**), 0.001(***).

The mechanisms of the impairing effects of Ni and Cd on biological systems is generation of reactive oxygen species (ROS) with subsequent oxidative injuries resulting also in mitochondrial dysfunction (**Belyaeva** *et al.*, **2012; Maiti** *et al.*, **2018**). However, despite the fact that mitochondria is a target site for heavy metal actions (**Belyaeva** *et al.*, **2008**), exact mechanisms of the metal-induced disturbances in mitochondrial function related to ATP production are not well

understood. Currently, we have found that *S. pombe* cells exposed to high concentrations of Cd^{2+} (400 µM) and Ni^{2+} (1 mM) displayed a significant increase in Ca^{2+} content in comparison to the untreated cells (**Pozgajova** *et al.*, **2019**). Moreover, the results showed an enhanced stimulating impact of Ni^{2+} treatment on intracellular Ca^{2+} level elevation as compared to Cd^{2+} exposure which is consistent with the current findings revealing different ATP production levels between both groups of treated cells. Regarding enhanced impact of Ni on ATP production compared to Cd we speculate that this fact could be attributed to higher tolerance of Ni by the yeast due to its crucial role in certain enzymes biosynthesis. In *S. pombe*, the metal is specifically incorporated into nickel-dependent enzyme such as urease (Ure2) catalyzing ATP-independent urea assimilation **(Milne** *et al*., **2015**). Generally, mitochondria of the most biological systems are capable of cytoplasmic Ca^{2+} sequestration to provide a transient calcium store for cell-protective mechanisms (**Baretto** *et al.*, **2014**). The research of **Jouaville** *et al.* (**1999**) showed that mitochondrial Ca^{2+} accumulation triggers an activation of the mitochondrial metabolic machinery leading to increased ATP synthesis in the organelle and consequently in the cytosol. Significantly increased concentrations of Ca^{2+} are invoked by the response of cells to heavy metal-induced excessive ROS generation subsequently stimulating Ca^{2+} release from the endoplasmic reticulum (ER) lumen followed by the massive influx of Ca^{2+} into the mitochondria. Although such Ca^{2+} overload is mostly associated with the induction of cell apoptosis (**Bayley** *et al.*, **2018**), Ca^{2+} overload also enhances mitochondrial activity leading to increased ATP production, causing along with decelerated metabolism, its accumulation within the cell. On the basis of all aspects we proposed that considerable higher levels of ATP detected in *S. pombe* cells exposed to Cd^{2+} and Ni^{2+} can be linked to the action of the heavy metal-induced ROS on cell (mitochondria) ion homeostasis.

Quantitation of ATP amounts becomes to have widespread implementation in various fields of studies such as physiology, metabolomics, or energetic studies. Moreover, over the last years the applications of luminescence measurements have been employed in ecological researches including microbial survival, microbial predation, plant pathogenicity, and reporting of gene expression in environmental samples (**Prosser** *et al*. **1996**). However, the most common and practical use of an ATP bioluminescence assay is to evaluate the microbial quality of the food industry through testing of solid surfaces cleanness as part of general hazard analysis and critical control points (HACCP) measures (**Carrascosa** *et al.* **2012**). With the use of previously described system of ATP measurement, we have evaluated the cleanness of the margarine production company after sanitation.

ATP bioluminescence control of the cleanness of margarine company equipment surfaces

Eleven samples from different parts of the margarine production line were analyzed using bioluminescence. As compared to the negative control, higher values for relative light or luminescence units (RLU) were recorded only in two evaluated samples. However, statistically significant differences ($p < 0.05$) have been found only between the control $\log_{10}$ relative luminescence units ($\log_{10}$ RLU 2.88±0.55) and $\log_{10}$ RLU of the sample Nr. 1 (4.84±0.11). This fact indicates a high level of cleanness of the margarine producing company devices except for a moderate contamination of the first representative sampling place. Calculated amounts of ATP in all investigated samples are shown in Table 2.

Table 2 Evaluation of cleanness as marker of sanitation efficiency of the devices used for margarine production. Data are expressed as ATP [nM] levels in case of RLU values greater than RLU values of the negative control. RLU values equal or lower as those of the negative control were assessed as negative and represent high level of cleanness.

Sample Nr.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
ATP [nM]	0.132±0.0047	0.00355±0.00013	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.

ATP: adenosine 5'-phosphate; SD: standard deviation

Detection of foodborne pathogens by bioluminescence have been applied in many studies (**Samkutty** *et al*., **2001**; **Leon & Albrecht**, **2007**; **Luo** *et al*., **2009**; **Hunter & Lim**, **2010**; **Lee** *et al*., **2017**). The method was also widely used for determination of the surface cleanliness in the food industry (**Vilar** *et al.*, **2008**; **Osimani** *et al.*, **2014**) and for monitoring of healthcare facilities (**Aycicek** *et al.*, **2006**; **Willis** *et al*., **2007**; **Boyce** *et al*., **2009**; **Fukada** *et al.*, **2015**). The results by **Osimani** *et al.* (**2014**) have revealed that ATP bioluminescence technology has proved to be a powerful tool for the real time monitoring of surface cleanliness at mass catering plants, to verify the correct application of Sanitation Standard Operating Procedures, and hence for their implementation/revision in the case of poor hygiene. The sensitivity, speed, and convenience of the evaluation process make this a powerful technique that is being applied in the risk assessment (**Carrascosa** *et al.* **2012**). While, most of the test laboratories use portable luminometers for the ATP content determination, we used non-portable Glomax Multi+ Combined Spectro-Fluoro Luminometer located in our laboratory to test the cleanness of the food producing company devices to approve suitability of the technique for such type of analyses. As the Glomax Multi+ Combined Spectro-Fluoro Luminometer is equipped with injectors allowing direct injection of the substrate separately to each sample prior to the measurement, acquired signal is almost free from any background noise, thus enabling very low ATP amount detection. We assume that the presented ATP detection system is applicable for cleanness validation of food processing companies, as well as for detection of microbial predation or indirect determination of mitochondrial activity of tested biological system under environmental stress.

CONCLUSION

According to our results, the luminescence-based system of ATP measurement provides fast, cost effective, and reliable technique for determination of microorganism presence in the tested sample. Moreover, the technique is capable of detection of mitochondrial activity alterations under environmental stress conditions. However, to understand the precise mechanism of ATP flux under environmental stress, further analyses are required. Nevertheless, the ability to detect considerably low amount of microorganism occurrence in the tested sample makes it a powerful technique for cleanness assessment after periodical sanitation of food processing companies.

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