Original Communication

Microbial cell autofluorescence as a method for measuring the intracellular content of B2 and B6 vitamins

Roman Maslanka, Michał Przywara, Agnieszka Janeczko, and Renata Zadrag-Tecza

Institute of Biology, College of Natural Sciences, University of Rzeszow, Poland

Abstract: Vitamins are important organic compound required for the proper functioning of cells and organisms. Vitamins of special industrial and pharmaceutical interests include riboflavin (vitamin B2) and pyridoxine (vitamin B6). Commercial production of those biological compounds has increasingly relied on microorganisms and requires simple methods for detecting and estimating their level of synthesis during the biotechnological process. In the case of yeast, methods based on autofluorescence, i.e. natural fluorescence emitted by several cellular compounds, including vitamins, may be useful. Considering that the intensity of emitted light is proportional to the intracellular concentration of riboflavin and pyridoxine, autofluorescence may be a convenient method for their quantification. In this report, we demonstrate a simple, rapid, and sufficiently trustworthy spectrofluorimetric method for determining the content of vitamins B2 and B6 in yeast cells which consists of cells growing, harvesting, washing, and resuspending in a buffer, and then measuring the emitted visible light using specific wavelength of excitation (λ_{ex}=340 nm and λ_{em}=385 nm for pyridoxine; λ_{ex}=460 nm and λ_{em}=535 nm for riboflavin). The limits of detection (LOD) and quantification (LOQ) estimated through measurements of vitamin fluorescence were below 0.005 μg/ml for riboflavin and below 0.05 μg/ml for pyridoxine, respectively. In turn, the smallest credible cell density for measuring autofluorescence was set at 1×10^8 yeast cells/ml. The relative level of the cell's autofluorescence can be expressed in mass units by applying proper calculation formulas. A comparison of the autofluorescence-based method with the reference HPLC-UV method shows that autofluorescence measurement can be used in the screening analysis of vitamin content (especially riboflavin) in microbial cells.

Keywords: autofluorescence, endogenous fluorophores, riboflavin, pyridoxine, yeast

Introduction

Vitamins are important organic compound required for the proper functioning of cells and organisms. Usually, they play a primary role as a cofactor for numerous enzymatic reactions. Water-soluble, and thus not easily stored, B vitamins are very important for several cellular redox reactions [1]. Although vitamins are essential for all living organisms, some taxa, especially humans and most animals, cannot synthesise *de novo* many vitamins, and therefore they have to obtain them from the environment, mainly through diet. Apart from physiological role and importance for human health, B vitamins are also intensively used as animal feed additives, cosmetics ingredients, or food colorants and antioxidants in food production [2]. Vitamins of special industrial and pharmaceutical interest are riboflavin (vitamin B2) and pyridoxine (vitamin B6). Riboflavin as a precursor for flavin coenzymes (FAD – flavin adenine dinucleotide and FMN – flavin mononucleotide) is a key component of the cellular biochemical processes. Flavin coenzymes are involved in the metabolism of carbohydrates, lipids, ketone bodies, and proteins [3, 4]. Additionally, riboflavin plays role in maintaining the redox state of Fe-S clusters [5, 6], and protects cells against oxidative stress by maintaining the proper intracellular level of reduced glutathione (GSH) or due to direct reactive oxygen species (ROS) scavenging properties [7, 8]. Vitamin B6, which comprises pyridoxine and its interconvertible forms, also exhibits antioxidant activities, connected with direct quenching of ROS, prevention of lipid peroxidation, or maintaining proper GSH content by stimulating its synthesis [9, 10]. What's more, vitamin B6 is involved in more than 140 different cellular metabolic reactions, *inter alia* it is important for: (i) proper folding and stabilization of particular proteins; (ii) biosynthesis and degradation of amino acids, mainly by its usage in transamination reactions; (iii) degradation of cellular storage compounds; or (iv) biosynthesis of epinephrine, dopamine, and serotonin, neurotransmitters connected with mental health [11, 12]. Vitamin B6 deficiency has been also associated with an increased level of DNA

damage, genome instability, and a higher risk of cancer development [12]. The high application potential of vitamins B2 and B6 is connected with a high demand for their production, which can be achieved by chemical or biological synthesis. Nowadays, driven by economic but also environmental-friendly considerations (i.e. reduction of carbon footprint, reduced usage of water, using renewable sources and food production waste), the commercial production of those vitamins (especially riboflavin) is moving toward the use of microbial fermentation [4, 13, 14]. The microorganisms used for industrial riboflavin production include *Bacillus subtilis*, *Ashbya gossypi*, *Candida famata* and many others promising microorganisms such as *Pichia guilliermondii* or *Hansenula polymorpha* [3, 4, 8]. There are also attempts to produce vitamin B6 using microorganisms such as *B. subtilis*, *P. guilliermondii*, or engineered strains of *Escherichia coli* [13]. For the last few years, attempts have been made to develop vitamin-overproducing microbial strains [4, 15]; however, optimisation of the fermentation process by modulating culture medium components, mainly carbon sources, has been equally important. This follows from the fact that the precursors for vitamins B2 and B6 are derived from central carbon metabolism (CCM), especially the pentose phosphate pathway (PP pathway) [15, 16]. Continuous improvement and optimization of biotechnological processes of vitamin synthesis with the use of microorganisms require proper methods for the detection of synthesized products. From an industrial perspective, such methods should be fast, simple, cost-effective, and as accurate as possible to estimate the level of desired products during the production/fermentation process. At present, due to their high sensitivity and high selectivity HPLC, UHPLC or HPLC-MS/MS have become the main analytical techniques used for the determination of vitamins [17]. Although liquid chromatography methods seem to be the most reliable and frequently used techniques, their main disadvantages are the complexity of equipment and high cost. Therefore, the development of screening methods, being low cost, having short analysis time, and needing usually the standard equipment, is also required. Such advantages are shown by spectrofluorimetry, which in combination with the fact that some water-soluble vitamins (including vitamin B2 and B6) manifest native fluorescence properties [16, 18], can be used as a screening method to detect vitamins in different samples. There are several reports presenting using of spectrofluorimetric methods for the determination of the vitamins in their pure or multivitamin pharmaceutical preparations [19, 20]. Yeast cells show a high level of autofluorescence (AF) associated with the presence of endogenous fluorophores, including tryptophan, and pyridine cofactors (NAD(P)H), but also pyridoxine, and riboflavin [16, 21]. Hence the question, of whether it is possible to determine the content of vitamins in yeast cells by measuring its autofluorescence *in situ*, arises.

In this report, we demonstrate a simple, rapid, and reliable spectrofluorimetric method for determining the content of riboflavin and pyridoxine in yeast cells, by measuring the relative level of cells autofluorescence and applying proper calculation formulas to express obtained values in a mass units.

Materials and methods

Chemicals

Vitamin B2 (riboflavin $\geq$ 98%); vitamin B6 (pyridoxine $\geq$ 98%) were from Sigma-Aldrich (Poznan, Poland). Components of culture media were from BD Difco (Becton Dickinson and Company, Spark, USA) except for glucose (POCH, Gliwice, Poland).

Yeast strains and growth conditions

The following yeast strains representing different genetic backgrounds were used: BY4741 *MAT*a *his3 leu2 met15 ura3* (EUROSCARF) and SP20 *MAT*a *leu1 ade1* [22]. Yeast was grown in the liquid YPD medium with 1% Yeast Extract, 1% Yeast Bacto-Peptone with 2% glucose concentrations on a rotary shaker at 150 rpm, at the temperature of 28 °C.

Determination of pyridoxine and riboflavin content in the yeast cells using the autofluorescence method

The fluorescence of yeast cells suspension was measured using the same parameters as was previously described by Maslanka et al., 2018 [16] and as was used for pure solutions of vitamins. Yeast cells from the late-exponential phase of growth were counted with the use of the Malassez chamber, then washed twice with sterile PBS with pH 7 and suspended to a final density of 1×10^8 cells/ml or 2×10^8 cells/ml in the same buffer. The 200 µl of cell suspensions were transferred to a microplate and the fluorescence was measured using the microplate reader Tecan Infinite 200 (Tecan Group Ltd., Männedorf, Switzerland) at the wavelengths λ_{ex}=340 nm and λ_{em}=385 nm for pyridoxine; λ_{ex}=460 nm and λ_{em}=535 nm for riboflavin. The values were expressed in the arbitrary unit but also were transformed to quantifiable values of vitamin concentration [µg/ml] using standard curves obtained from the fluorescence of known vitamin concentrations. Limit of detection (LOD) and limit of quantification (LOQ) for fluorescence detection of vitamin B2 and B6 where determined from the parameters of

the calibration curves and expressed in μg/ml. LOD and LOQ were determined using the formulas LOD=[3.3×(σ/a)] and LOQ=[10×(σ/a)]; where σ – standard deviation of the y-intercept, a – slope of the calibration curve. The number of the yeast cells using in the autofluorescence method (density of 1×10^8 cells/ml or 2×10^8 cells/ml) where selected with regard to vitamin limits of detections and quantification. The precision of autofluorescence determination in the yeast cells at the wavelengths characteristic to pyridoxine and riboflavin was measured by the coefficient of variation (CV) calculated from nine results obtained for each yeast strain (i.e. three technical replicates from three independent biological replicates). Knowing the number of cells in cell suspension and the weight of the specified number of cells, content of analyzed vitamins was expressed in the form of mg per 100g yeast cell mass.

Determination of pyridoxine and riboflavin content in the yeast cells using the HPLC-UV method – validation the accuracy of the autofluorescence method

To determine accuracy of the autofluorescence (AF) method the obtained results of AF were compared with the results received by HPLC method. Vitamins were determined according to JARS, Poland, certificate of the Polish Center for Accreditation no AB 1095. The quantification of vitamin riboflavin and pyridoxine was performed using a high-performance liquid chromatography method with spectrophotometric detection (HPLC-UV). Test procedure by the method PB-257/LF edition 4 dated 30 May 2021.

Statistical analysis

Results are presented as mean±SD from at least three independent experiments. The statistical analysis was performed using STATISTICA 10.0 software. The statistical significance of the differences between the means obtained from analyzed yeast strains was estimated using the *t-Student's* test. The values were considered significant at $p < 0.05$. Used designation: *a* – different to 1×10^8 cells/ml density; *b* – different to 2×10^8 cells/ml density; * – differences between strains within the same number of cells in cell suspension.

Results

Quantitative determination of vitamins by measurement of the autofluorescence

Given the growing interest in measuring autofluorescence as an analytical method, we tested if autofluorescence can be used as a simple and enough precise method for the quantitative determination of the intracellularly produced B vitamins. For that purpose, the autofluorescence measurements of yeast cell suspension and the determination of vitamin content using a fluorescence calibration curve of known concentrations of pure vitamin solutions were performed. The calculated from calibration curve fluorescence limits of detection (LOD) and quantification (LOQ) were 0.001 and 0.003 μg/ml for riboflavin and 0.014 and 0.042 μg/ml for pyridoxine, respectively. The autofluorescence measurements in yeast cells were done for two different yeast strains (SP20 and BY4741) and different numbers of cells in the suspension. The usefulness, accuracy and validation of the proposed autofluorescence-based method were checked by comparison of vitamin content in yeast obtained by autofluorescence with the results obtained with the reference HPLC-UV method. Under the prevalent conditions, the optimum excitation and emission wavelengths for riboflavin (ex. at 460 nm and em. at 535 nm) and pyridoxine (ex. at 340 nm and em. at 385 nm) were the same as we noted previously [16] and very similar to those noted in the literature data [23, 24]. The obtained results showed that the SP20 strain had a higher level of autofluorescence than values observed for the BY4741 strain (Figures 1A and 1B). These were observed both for levels of autofluorescence corresponding to pyridoxine and riboflavin fluorescence (Figures 1A and 1B). The noted values of autofluorescence were strictly dependent on the number of yeast cells (Figures 1A and 1B). The suspension with a density of 2×10^8 cells per ml has appropriately higher autofluorescence levels than the suspension with a density of 1×10^8 cells per ml. The values of autofluorescence converted based on calibration curves (Figures 1C and 1D) on the particular content of pyridoxine and riboflavin showed the same relation (Figures 1E and 1F). The precision of autofluorescence determination in the yeast cells was checked by analyzing repeatability and intermediate precision. Repeatability was checked by measuring the coefficient of variation (CV) of the calculated concentrations from the technical replicates and the intermediate precision was analyzed by measuring CV considering technical and biological replicates. In the case of the BY4741 strain the CV relating to repeatability averaged 2.99% for riboflavin and 5.14% for pyridoxine, respectively. Those CV values in the case of the SP20 strain averaged 2.14% for riboflavin and 2.45% for pyridoxine, respectively. CV for intermediate precision averaged 6.13% for riboflavin and 8.04% for pyridoxine in the case of the BY4741 strain and 2.94% for riboflavin and 4.24% for pyridoxine in the case of the SP20 strain, respectively. The received values of CV are within acceptable ranges, so autofluorescence measurements of the yeast cells can be considered as precise.

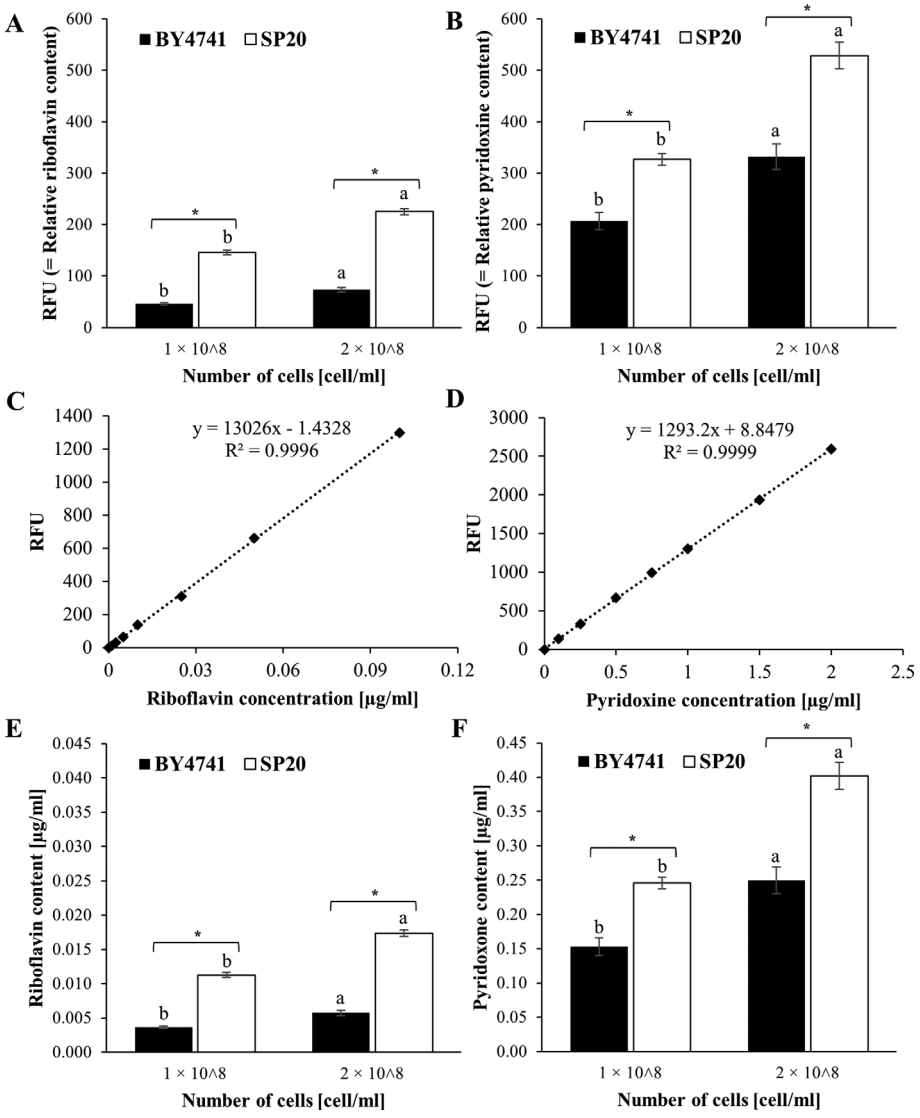

Figure 1. The riboflavin and pyridoxine content in the different wild-type yeast strains cells determined by autofluorescence measurement. Fluorescence intensity measured at wavelength characteristic to riboflavin fluorescence (A); Fluorescence intensity measured at wavelength characteristic to pyridoxine fluorescence (B); Calibration curve of riboflavin (C) and pyridoxine (D) performed for pure compounds dissolved in ultrapure water; Content of riboflavin (E) and pyridoxine (F) expressed in µg/ml based on calibration curves. Error bars indicate SD from at least three independent experiments in which three measurements of the sample were performed. The values were considered significant at $p<0.05$. The letters *a* and *b* indicate the differences between values observed in suspension with a different number of yeast cells. [a]Different to 1×10^8 cells/ml density; [b]different to 2×10^8 cells/ml density; *differences between BY4741 and SP20 strains within the same number of cells in the cell suspension.

The validation of the vitamin content in the mass units

In order to check and validate the vitamin content in the samples, the obtained results were converted into the form of mg of vitamins per 100g of yeast biomass. It was observed that the content of vitamins expressed in mg/100g was almost identical, no matter what density of the culture was taken for the determination (Figure 2). The pyridoxine content in yeast cells was higher than riboflavin in both analyzed yeast strains (Figure 2A and 2B), which is in line with data on nutrient composition in yeast. The comparison between strains shows that the SP20 strain had about three times higher content of riboflavin and almost two times higher content of pyridoxine than the contents of those vitamins noted in the BY4741 strain (Figure 2A and 2B).

Comparison of the results of quantitative determination of vitamins by measurement of the autofluorescence and HPLC-UV method

Expressing the results in mg/100g units is handier from a technological point of view; it also allows for comparing

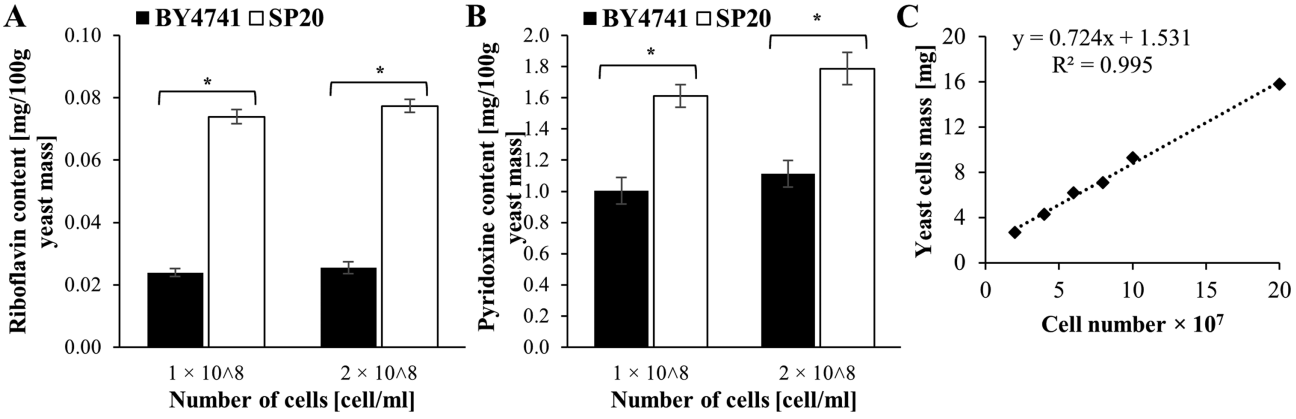

Figure 2. Normalized content of analyzed vitamins in the different wild-type yeast strains cells. The riboflavin (A) and pyridoxine (B) content were expressed in the form of mg per 100g yeast cell mass. The mass of the yeast cells was indicated based on a calibration curve (C) prepared by measurements of the weight of the specified number of cells. Error bars indicate SD from at least three independent experiments in which three measurements of the sample were performed. The values were considered significant at $p < 0.05$. *Indicate the differences between BY4741 and SP20 strains within the same number of cells in the cell suspension.

Table 1. Content of riboflavin and pyridoxine in the analysed yeast strains measured by autofluorescence (AF) and HPLC-UV methods and the way normalization of results for the inter-comparability of results between different methods

				The ratio of vitamin content between SP20/BY471		Conversion factor (CF)	Normalized conversion factor
Vitamin content	Yeast strain	AF method [mg/100 g]	HPLC method [mg/100 g]	AF method	HPLC method	AF/HPLC ratio	Average of CF
Riboflavin	BY4741	0.0247*±0.0015	0.0118*±0.0014	3.06±0.03	3.39±0.02	2.10±0.07	2.0±0.1
	SP20	0.0757*±0.0022	0.0400*±0.0050			1.91±0.04	
Pyridoxine	BY4741	1.0577*±0.0849	0.0393*±0.0047	1.61±0.01	2.19±0.01	26.91±1.37	23.3±4.1
	SP20	1.6998*±0.0882	0.0860*±0.0100			19.76±1.02	

Notes. Results are presented as mean±SD from at least three independent experiments with three technical replicates each in the case of autofluorescence measurements and from at least two biological replicates in the case of HPLC-UV determination. The values were considered significant at $p<0.05$. *Indicates the differences between BY4741 and SP20 strains within the content of vitamins. CF – conversion factor indicating the number used to change one another values obtained by AF or HPLC-UV, by multiplying or dividing, was calculated individually for each strain and vitamins. Normalized CF was determined on the basis of all values of CF obtained for each vitamin. Normalized CF can be treated as an autofluorescence method coefficient.

and analysing accuracy of the results against those obtained through the reference HPLC-UV method. Results of HPLC-UV confirmed the dependences observed with autofluorescence measurements, that in yeast cells content of pyridoxine is higher than the content of riboflavin, but also that the content of vitamins in the SP20 strain is higher than those noted for the BY4741 strain. The content of riboflavin and pyridoxine in SP20 was about three times higher and almost two times higher than the contents of those vitamins noted in the BY4741 strain, respectively (Table 1). Although, in the case of direct comparison of those two methods by the ranges of obtained results, there were some differences. For riboflavin, the results were of the same order of magnitude, but for pyridoxine, the differences in the obtained levels were much greater (Table 1). Hence the accuracy of autofluorescence method is not without drawbacks, especially in the context of pyridoxine determination, but can be adapted for simple semi-quantitative screening tests indicating changes in intracellular content of vitamins.

Discussion

Autofluorescence – origin and applications for research purposes

Due to their high sensitivity, fluorescence assays are frequently used as analytical methods applied in biochemistry and cell biology. In most cases, those assays use external fluorophores to visualize non-fluorescent cell compounds or obtain information on the cells' physiological status. However, there are also many compounds (endogenous fluorophores) that are responsible for autofluorescence i.e. intrinsic natural fluorescence of biological samples. Those compounds are widely distributed in all types of cells and include aromatic amino acids (Phe, Trp, Tyr), vitamins (B1, B2, B6), coenzymes and electron acceptors (FAD; NAD(P)H)) [18, 25]. The greatest abundance of molecules with autofluorescent properties is noted in plant cells. Apart from chlorophylls, they include carotenoids, several cell

wall components and a large group of secondary metabolites [18, 26]. Autofluorescence of human cells (apart amino acids, coenzymes and vitamins) can also be derived from compounds accumulated with age or during several disorders, e.g. lipofuscin formed as a result of lipid peroxidation or porphyrins accumulated during impaired heme synthesis [18, 25]. In the case of *Saccharomyces cerevisiae* yeast cells, autofluorescence is generally associated with the presence of tryptophan, pyridoxine, flavins, and NAD(P)H [16, 23, 27, 28]. Those molecules play an important role in cell metabolism and changes in their amount provide information about the physiological status of the cells. Therefore, determination of the autofluorescence level can be used for various analytical purposes, thus changing the attitude towards autofluorescence, which for a long time was regarded as an undesirable property only. The use of autofluorescence for research purposes presents several advantages: (i) autofluorescence-based techniques do not require any additional steps such as staining or fixation; (ii) no introduction of chemicals decreased the toxicity for sample, user, and environment, but also lowers the costs of measurements; (iii) nonspecific binding of chemicals and artefactual results are avoided; (iv) measurements can be performed *in situ* in native biological samples without disrupting their structures [18]. Because autofluorescence depends on cellular morphology as well as the metabolic and physiological state of cells, autofluorescence-based techniques can be used in diagnostics and medical applications. Till now, they are used among others for monitoring drug uptake and their distribution, discrimination between normal and pathological (such as cancer) cells, and monitoring of retinal disease by determination of fundus autofluorescence [18, 29]. In the case of microbiological studies, tryptophan autofluorescence is commonly used as an indicator of cell mass increase in the culture [23, 27]. Determination of cell autofluorescence is useful for the differentiation of microbial organisms as well as for strain characterization [16, 30, 31, 32]. Recently, yeast autofluorescence measurements led to proposes of the yeast-based biosensor to determinate labile Zn^{2+} in an aqueous solution [33].

The possible components of obtained autofluorescence values

Due to its cost-effectiveness, autofluorescence can be also used for industrial purposes such as monitoring the effectiveness of biotechnological processes. It can be especially useful in the case of microbial biotechnological production of vitamins. For that purpose, methodological analysis and method validation using *S. cerevisiae* was tested. The comparison of results obtained by autofluorescence measurement and HPLC-UV method showed that the conversion factor was almost identical and amounted to approx. 2 for values of riboflavin and approx. 20–25 for values of pyridoxine (Table 1). Substantial differences in the amount of pyridoxine between methods may arise from the fact that biological samples contain a mixture of many native fluorophores. Especially in the case of purple and blue spectrum of emission, there are several cellular compounds whose spectra may overlap [24]. One of the interfering compounds can be NAD(P)H, whose excitation maximum is close to the excitation wavelength for pyridoxine. Although the emission maximum of this major electron acceptor is around 450–460 nm and is separated from the emission maximum of pyridoxine [18, 25], part of fluorescence emitted by NAD(P)H even in the shorter-than-maximum emission wavelength may inflate the results of pyridoxine measurement. A similar influence can be caused by another yeast endogenic fluorophore, ergosterol. Ergosterol is a typical sterol component of the cell membrane in fungi and thus can be involved in the proliferation of yeast cells. Its fluorescence properties are close to those of pyridoxine, especially in the case of excitation wavelength (360–370 nm region is found specific to ergosterol detection). However, the ergosterol fluorescence emission spectral interval is not so specific and is in the range of 400–600 nm [34]. The values of pyridoxine can be also overestimated by fluorescence of cell wall components, such as chitin [35]. The values obtained in the case of riboflavin content were more precise, which can be connected with the fact that fluorescence emitted by flavins is in a different part of the wavelength spectrum, and flavins are treated as one of the major contributors to cell autofluorescence. Hence, they seem to be more appropriate for determining differences between cells. For that reason, the autofluorescence properties of flavin are used more and more frequently to determine cell energy metabolism, which can be useful not only for yeast or bacterial cells but also in the case of human cells [36].

The advantages and limitations of method based on autofluorescence measurement

The results obtained in this report show that usage of autofluorescence-based methods (especially in the context of riboflavin) can be widely applied also as a screening tool for the detection of vitamins synthesized in microbial fermentation. This seems to be particularly useful for vitamins-overproducing microbial strains and optimization of the fermentation process. Cellular biosynthesis of riboflavin and pyridoxine is directly dependent on CCM [16, 37], and the pentose phosphate (PP) pathway seems to play the most important role here, which is confirmed by studies revealing that overexpression of enzymes involved in the PP pathway increases the carbon flux through that pathway and simultaneously enhances riboflavin production [4, 15, 38, 39]. The obtained results and proposed methodology

allows to monitoring the intracellular level of riboflavin and expand the range of possibilities to measure vitamins content through fluorescence analyses, which present are mainly determined in the culture medium [5]. The results presented in this report demonstrate that analysis based on autofluorescence measurements can be successfully used to estimate the content of vitamins in yeast cells, especially concerning riboflavin content. It should be noted however that autofluorescence can also be affected by other factors, such as specific cellular constituents, which show autofluorescence in a range of excitation similar to that of the analysed vitamins. This fact should be taken into account when interpreting the results. However, by comparing the proposed methodology with the reference HPLC-UV method, we show that despite these limitations the autofluorescence-based techniques can be used in the screening analysis of vitamin content in microbial cells. Additionally, fast, simple, and cost-effective determination of vitamins in the proposed method are advantages especially useful in biotechnological processes of vitamin synthesis with the use of microorganisms. It also gives the possibility of more effective and faster verification of the results of changes introduced during the biotechnology process.

In summary, microbial cell autofluorescence can be used as a non-invasive and non-destructive method for measuring the intracellular content of riboflavin and pyridoxine. Monitoring the autofluorescence of yeast cells is a quick and effective method for measuring the intracellular content of these vitamins and for that reason can be useful in a range of applications, including biotechnology, food science, and nutrition research.

References

1. Lykstad J, Sharma S. Biochemistry, water soluble vitamins [Internet]. Treasure Island (FL): StatPearls Publishing. 2022.
2. Revuelta JL, Buey RM, Ledesma-Amaro R, Vandamme EJ. Microbial biotechnology for the synthesis of (pro)vitamins, biopigments and antioxidants: challenges and opportunities. Microb Biotechnol. 2016;9(5):564–7.
3. Abbas CA, Sibirny AA. Genetic control of biosynthesis and transport of riboflavin and flavin nucleotides and construction of robust biotechnological producers. Microbiol Mol Biol Rev. 2011;75(2):321–60.
4. Averianova LA, Balabanova LA, Son OM, Podvolotskaya AB, Tekutyeva LA. Production of vitamin B2 (riboflavin) by microorganisms: an overview. Front Bioeng Biotechnol. 2020;8:570828.
5. Demuyser L, Palmans I, Vandecruys P, Van Dijck P. Molecular elucidation of riboflavin production and regulation in candida albicans, toward a novel antifungal drug target. mSphere. 2020;5(4):e00714–20.
6. Gnandt E, Dörner K, Strampraad MFJ, de Vries S, Friedrich T. The multitude of iron-sulfur clusters in respiratory complex I. Biochim Biophys Acta. 2016;1857(8):1068–72.
7. Ashoori M, Saedisomeolia A. Riboflavin (vitamin B2) and oxidative stress: a review. Br J Nutr. 2014;111(11):1985–91.
8. Blazhenko OV. Glutathione deficiency leads to riboflavin oversynthesis in the yeast *Pichia guilliermondii*. Curr Microbiol. 2014;69(1):10–8.
9. Bilski P, Li MY, Ehrenshaft M, Daub ME, Chignell CF. Vitamin B6 (pyridoxine) and its derivatives are efficient singlet oxygen quenchers and potential fungal antioxidants. Photochem Photobiol. 2000;71(2):129–34.
10. Chumnantana R, Yokochi N, Yagi T. Vitamin B6 compounds prevent the death of yeast cells due to menadione, a reactive oxygen generator. Biochim Biophys Acta. 2005;1722(1):84–91.
11. Parra M, Stahl S, Hellmann H. Vitamin B6 and its role in cell metabolism and physiology. Cells. 2018;7(7):84.
12. Wu XY, Lu L. Vitamin B6 deficiency, genome instability and cancer. Asian Pac J Cancer Prev. 2012;13(11):5333–8.
13. Rosenberg J, Ischebeck T, Commichau FM. Vitamin B6 metabolism in microbes and approaches for fermentative production. Biotechnol Adv. 2017;35(1):31–40.
14. Stahmann KP, Revuelta JL, Seulberger H. Three biotechnical processes using *Ashbya gossypii*, *Candida famata*, or *Bacillus subtilis* compete with chemical riboflavin production. Appl Microbiol Biotechnol. 2000;53(5):509–16.
15. Liu S, Hu W, Wang Z, Chen T. Production of riboflavin and related cofactors by biotechnological processes. Microb Cell Fact. 2020;19(1):31.
16. Maslanka R, Kwolek-Mirek M, Zadrag-Tecza R. Autofluorescence of yeast *Saccharomyces cerevisiae* cells caused by glucose metabolism products and its methodological implications. J Microbiol Methods. 2018;146:55–60.
17. Zhang Y, Zhou WE, Yan JQ, Liu M, Zhou Y, Shen X, et al. A review of the extraction and determination methods of thirteen essential vitamins to the human body: an update from 2010. Molecules. 2018;23(6):1484.
18. Monici M. Cell and tissue autofluorescence research and diagnostic applications. Biotechnol Annu Rev. 2005;11:227–56.
19. Barary M, Abdel-Hamid M, Hassan E, Elsayed M. Simultaneous spectrofluorimetric determination of thiamin, pyridoxine and riboflavin in pharmaceutical multivitamin preparations. Pharmazie. 1986;41(7):483–5.
20. Mohamed AM, Mohamed HA, Abdel-Latif NM, Mohamed MR. Spectrofluorimetric determination of some water-soluble vitamins. J AOAC Int. 2011;94(6):1758–69.
21. Kwolek-Mirek M, Maślanka R, Molon M. Disorders in NADPH generation via pentose phosphate pathway influence the reproductive potential of the Saccharomyces cerevisiae yeast due to changes in redox status. J Cell Biochem. 2019;120:8521–33.
22. Biliński T, Litwińska J, Sledziewski A, Rytka J. Hemoprotein formation in yeast. VII. Genetic analysis of pleiotropic mutants affected in the response to glucose repression and anoxia. Acta Microbiol Pol. 1980;29(3):199–212.
23. Horvath JJ, Glazier SA, Spangler CJ. In situ fluorescence cell mass measurements of *Saccharomyces cerevisiae* using cellular tryptophan. Biotechnol Prog. 1993;9(6):666–70.
24. Wagnières GA, Star WM, Wilson BC. In vivo fluorescence spectroscopy and imaging for oncological applications. Photochem Photobiol. 1998;68(5):603–32.
25. Andersson H, Baechi T, Hoechl M, Richter C. Autofluorescence of living cells. J Microsc. 1998;191(Pt 1):1–7.
26. Talamond P, Verdeil JL, Conéjéro G. Secondary metabolite localization by autofluorescence in living plant cells. Molecules. 2015;20(3):5024–37.
27. Podrazký O, Kuncová G, Krasowska A, Sigler K. Monitoring the growth and stress responses of yeast cells by two-dimensional

fluorescence spectroscopy: first results. Folia Microbiol (Praha). 2003;48(2):189–92.
28. Siano SA, Mutharasan R. NADH and flavin fluorescence responses of starved yeast cultures to substrate additions. Biotechnol Bioeng. 1989;34(5):660–70.
29. Yung M, Klufas MA, Sarraf D. Clinical applications of fundus autofluorescence in retinal disease. Int J Retina Vitreous. 2016;2:12.
30. Bhatta H, Goldys EM. Characterization of yeast strains by fluorescence lifetime imaging microscopy. FEMS Yeast Res. 2008;8(1):81–7.
31. Bhatta H, Goldys EM, Learmonth RP. Use of fluorescence spectroscopy to differentiate yeast and bacterial cells. Appl Microbiol Biotechnol. 2006;71(1):121–6.
32. Maślanka R, Zadrag-Tecza R. Different life strategies in genetic backgrounds of the *Saccharomyces cerevisiae* yeast cells. Fungal Biol. 2022;126(8):498–510.
33. Sun A, Wang WX. Adenine deficient yeast: a fluorescent biosensor for the detection of Labile Zn(II) in aqueous solution. Biosens Bioelectron. 2021;179:113075.
34. Croce AC. Light and autofluorescence, multitasking features in living organisms. Photochem. 2021;1(2):67–124.
35. Krishnamoorthy R, Gassem MA, Athinarayanan J, Periyasamy VS, Prasad S, Alshatwi AA. Antifungal activity of nanoemulsion from *Cleome viscosa* essential oil against food-borne pathogenic *Candida albicans*. Saudi J Biol Sci. 2021;28(1):286–93.
36. Surre J, Saint-Ruf C, Collin V, Orenga S, Ramjeet M, Matic I. Strong increase in the autofluorescence of cells signals struggle for survival. Sci Rep. 2018;8(1):12088.
37. Cohen GN, Microbial Biochemistry. Second ed. London New York: Springer Science+Business Media B.V; 2011.
38. Duan YX, Chen T, Chen X, Zhao XM. Overexpression of glucose-6-phosphate dehydrogenase enhances riboflavin production in *Bacillus subtilis*. Appl Microbiol Biotechnol. 2010;85(6):1907–14.
39. Wang Z, Chen T, Ma X, Shen Z, Zhao X. Enhancement of riboflavin production with *Bacillus subtilis* by expression and site-directed mutagenesis of zwf and gnd gene from *Corynebacterium glutamicum*. Bioresour Technol. 2011;102(4):3934–40.

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ORCID
Roman Maslanka
https://orcid.org/0000-0003-1917-4222
Michał Przywara
https://orcid.org/0000-0002-8942-0866
Agnieszka Janeczko
https://orcid.org/0000-0002-5674-5656
Renata Zadrag-Tecza
https://orcid.org/0000-0001-5073-9274

Roman Maslanka
Institute of Biology
College of Natural Sciences
Univeristy of Rzeszow
Zelwerowicza 4
35-601 Rzeszow
Poland
rmaslanka@ur.edu.pl