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The use of LipidGreen2 for visualization and quantification of intracellular Poly(3-hydroxybutyrate) in *Cupriavidus necator*

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ABSTRACT

Numerous studies have been conducted to develop a rapid protocol for the quantification of poly(3-hydroxybutyrate) during bacterial fermentation as an alternative to time-consuming gravimetric or analytical methods. Fluorescence spectroscopy is one of the most promising approaches. In this study, it could be demonstrated that the novel fluorescent probe LipidGreen2 is able to stain selectively poly(3-hydroxybutyrate) in *Cupriavidus necator*. Optimal excitation and emission wavelengths were evaluated using 3D-Excitation-Emission-Matrix, displaying the best intensities between 440-460 nm and 490–520 nm for excitation and emission, respectively. The lipophilic fluorophore LipidGreen2 showed a high long-term stability even when incubated under ambient lighting. Due to a strong linear relationship between side scatter and biomass concentration, the influence of the inner filter effects could be incorporated, and adjusting the sample to a specific OD is thus superfluous. The developed method allows a very accurate quantification of poly(3-hydroxybutyrate) in just 15 min, following a comprehensible and simple protocol. It is also excellently suited for bioimaging of intracellular poly(3-hydroxybutyrate) granules.

1. Introduction

Microorganisms represent an important renewable resource for high-quality lipids. This includes the biopolymer class of polyhydroxyalkanoates (PHA), which are polyesters of various hydroxyalkanoates. PHA are synthesized as an intracellular carbon and energy storage compound by many archaea as well as numerous gram-negative and gram-positive bacteria living in various habitats and are accumulated as granules in the cytoplasm of cells [1,2].The biodegradability of these polyesters makes them extremely desirable substitutes for conventional petroleum-based plastics [3,4]. The best-studied representative is poly(3-hydroxybutyrate) (PHB) that can be synthesized by model-organism *Cupriavidus necator* [5]. However, the main disadvantage of biotechnological production is the high manufacturing costs. In order to overcome this bottleneck, various approaches such as continuous production, solid state cultivation or mixed cultures with different types of substrates or the use of waste materials were investigated [6–9]. Detection and quantification of PHB are of great importance for determining the optimal harvest times. Analysis of PHB is predominantly performed by chromatographic and gravimetric methods [10,11]. However, these methods are usually time and material consuming and not suitable for online monitoring. Fluorescence spectroscopy presents an alternative since storage lipids can be quantified due to fluorochromination [12]. With lipophilic fluorescent dyes, PHB can be detected indirectly through the fluorescence emission in a short time. The most used and best-studied stains are Nile Red and BODIPY [13–16]. Solvatochromic dyes like Nile Red have the advantage of low background fluorescence, as the emission spectrum is shifted to blue during accumulation inside the PHB granules [17,18]. Nevertheless, Nile Red shows low sensitivity, coloring both cell membrane and storage lipids, and exhibits quenching effects at higher concentrations, making it challenging to create a reproducible and accurate quantification method [19,20].

In contrast, BODIPY shows a very sharp emission band and a very high quantum yield [21]. However, it has no solvatochromic properties. The resulting high background fluorescence makes quantification without further washing steps complicated [22].

Recently, a new synthesized solvatochromic small fluorescent molecule, namely LipidGreen, consisting of an indolin-3-one scaffold, has been used successfully for the selective staining of lipid droplets in cell lines and for PHB screening *in E. coli* mutant libraries [23,24]. Based on LipidGreen, the chemical derivative LipidGreen2 was developed,

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which compared to BODIPY showed better sensitivity and fluorescence efficiency with less background fluorescence. LipidGreen2 was also successfully used for the selective staining of fat reserves in zebrafish [25]. The structure and properties of LipidGreen and LipidGreen2, in contrast to Nile Red are given in Table 1.

The aim of our investigation is to develop a stable and reproducible fluorescence-based quantification method for storage lipids especially PHB, which can be used for online monitoring during the biotechnological cultivation of microorganisms. In this study, we present for the first time optimal staining conditions for a LipidGreen2 based detection and demonstrate the use for bioimaging of intracellular PHB granules. We have further developed an accurate quantification method for the model organism *Cupriavidus necator*.

2. Material and methods

2.1. Chemicals

LipidGreen2 - Calbiochem was purchased from Sigma-Aldrich/Merck (Darmstadt, Germany). Other chemicals were analytical grade and were obtained either from Carl Roth (Karlsruhe, Germany) or Sigma-Aldrich/Merck.

2.2. Strains and cultivation conditions

Production of PHB was conducted by culturing *Cupriavidus necator* DSM 428 obtained from the German Collection of Microorganisms and Cell Cultures. The start culture was grown in 100 mL nutrient-rich (NR) medium, containing 5 g L^{-1} of peptone and 3 g L^{-1} of meat extract, and agitated at 180 rpm for 48 h at 30 °C. The culture was then transferred to the seed culture medium for 48 h and retransferred again to the batch culture medium slightly modified, according to Ryu et al. [26]. After 48 h fermentation, the initial glucose/fructose substrate concentration was replenished to obtain high yields of PHB. Batch cultivation was carried out at 30 °C and agitated at 250 rpm in a simple 2,0 L aerated bioreactor. Media compositions are given in Table 2. The trace element solution consisted of 10 g L^{-1} $FeSO_4(7H_2O)$, 2.25 g L^{-1} $ZnSO_4(7H_2O)$, 1 g L^{-1} $CuSO_4(5H_2O)$, 0.5 g L^{-1} $MnSO_4(5H_2O)$, 2 g L^{-1} $CaCl_2(2H_2O)$, 0.23 g L^{-1} $Na_2B_4O_7(7H_2O)$, 0.1 g L^{-1} $(NH_4)_6Mo_7O_{24}$, and 10 mL L^{-1} 35% HCl [27].

2.3. Staining procedure

The LipidGeeen2 (LG2) staining procedure is based on the findings of Chun et al. [25]. A stock solution of 54 μM LG2 in dimethylsulfoxide (DMSO) was used for all assays. 500 μL of cell suspension was diluted in 1500 μL phosphate-buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 at pH 7.4. Afterward, 100 μL LG2 stock were added, obtaining a final LG2 concentration of 2.6 μM. Incubation took place at room temperature at different incubation times.

Table 2
Media composition for *Cupriavidus necator*.

Component	Seed Culture	Batch Culture
Na_2HPO_4 ($12H_2O$)	9.0 g L^{-1}	9.0 g L^{-1}
KH_2PO_4	1.5 g L^{-1}	1.5 g L^{-1}
$(NH_4)_2SO_4$	1.0 g L^{-1}	0.5 g L^{-1}
$MgSO_4$ ($7H_2O$)	0.2 g L^{-1}	0.2 g L^{-1}
Citric acid	1,7 g L^{-1}	1,7 g L^{-1}
Trace element solution	1 mL	10 mL
Glucose/Fructose	5/5 g L^{-1}	10/10 g L^{-1}

2.4. Fluorescence spectroscopy

Fluorescence measurements were performed with a PerkinElmer LS45 (Waltham, USA) using standard cuvettes with 1.0 cm path length (Brand, Wertheim, Germany). To distinguish optimal excitation and emission wavelengths, 3D-Excitation-Emission (3DEEM) spectra were obtained using wavelengths between 400 and 600 nm. For all further experiments, the excitation was fixed to 440 and emission to 505 nm using slits set to 10 nm and a gain of 650 mV. Side scatter measurements of the samples were performed before adding LG2 to the sample using the same parameters as described above.

2.5. Effect of time and lighting on the staining procedure

To investigate the photostability of LG2 during the staining process, light intensities of 0 μmol m^{-2} s^{-1} (light exclusion, dark), 10 μmol m^{-2} s^{-1} (ambient light), and 100 μmol m^{-2} s^{-1} (intense light) were chosen. Fluorescence was followed over different incubation times. A full sunlight spectrum LED lamp was used for the experiment. Long-term measurements were carried out using a 1 g L^{-1} LG2 stained cell culture over a period of 10 h at a measurement rate of 60 h^{-1} with a previous incubation for 30 min under light exclusion.

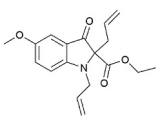
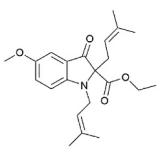
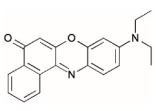
2.6. Epi-fluorescence microscopy

For visualization of intracellularly accumulated PHB, LG2 fluorescence was observed with a fluorescence microscope (Olympus BX41, Tokyo, Japan) equipped for epi-illumination by excitation at 400–440 nm and emission at 460–490 nm. Images were captured with an Olympus XC50 camera and analyzed with the cellSens Standard software.

2.7. Biomass determination

Growth was followed by gravimetry. Briefly, biomass samples were centrifuged (Thermo Scientific, Waltham, USA) at 5000×*g* for 10 min in micro reaction tubes (VWR, Darmstadt, Germany), washed twice with distilled water, frozen, lyophilized (Christ Martin, Osterrode, Germany) and weighed. Biomass concentration was calculated from the ratio of dry biomass to the cell culture volume used.

Table 1
Structure and properties of LipidGreen and LipidGreen2 in contrast to Nile Red. Properties were calculated with ChemDraw Ultra 11.0.

	LipidGreen	LipidGreen2	Nile Red
Structure			
Mol. Weight [DA]	315.4	371.2	318.1
Target	Lipid Globules	Lipid Globules	Lipid Globules/Plasma Membrane
Range Ex/Em [nm]	450/510	440/500-510	480-530/575-620
Hydrophobicity (clogP)	4.07	5.92	4.62

2.8. Analytical quantification of PHB

PHB concentration was detected by HPLC, according to Karr et al. [28] using the modified procedure as follows: 1000 μL of 75% sulfuric acid was added to the retained dried biomass and heated at 95 °C for 60 min. Dehydrated biomass was diluted ten times with distilled water before measurement. Solid PHB standard was treated alike and used for calibration. Quantification of crotonic acid (as the hydrolysis product of PHB) was assayed using a Merck-Hitachi HPLC with a UV detector at 214 nm and an ABOA SugarSep column (AppliChrom, Oranienburg, Germany). Elution was performed with 0.007 N H_2SO_4 at a flow rate of 0.8 mL min^{-1}, 60 °C, and 50 bar.

3. Results and discussion

3.1. Determination of excitation and emission wavelengths for LipidGreen2-based PHB quantification and bioimaging

Optimal excitation and emission wavelengths were studied through 3D-Excitation-Emission-Matrix (3DEEM) to determine suitable wavelength pairs, which can be used to analyze the fluorescence of PHB in *C. necator*. 1 mg mL^{-1} of stained cell culture was scanned at wavelengths ranging from 400 to 600 nm. Based on Fig. 1A, ideal excitation between 440 and 460 nm, and emission between 490 and 520 nm were observed. These results confirm the finding of Choi et al. [23], who used 450 nm and 510 nm for excitation and emission of LipidGreen1, respectively. In order to take into account a significant Stokes shift, an excitation wavelength of 440 nm and emission of 505 nm were chosen for subsequent experiments.

Under the same conditions, epi-fluorescence microscopic pictures were taken. PHB stained with LipidGreen2 showed an evident green fluorescence using an excitation of 400–440 nm that was stable over a period of several minutes (Fig. 1B). At longer excitation wavelength (460–490 nm), lifespan increased to more than 20 min. In summary, Lipidgreen2 can be used for bioimaging of intracellular PHB, but a longer excitation wavelength should be applied due to the rapid bleaching at short wavelength.

However, no staining of the cell membrane could be detected. Therefore, it can be assumed that LipidGreen2 has a higher affinity for PHB than Nil Red, which also stains cell membranes and shows a biphasic rise of fluorescence [20,21]. The difference could be explained by the higher hydrophobicity index of LipidGreen2 (Table 1).

3.2. Definition of LipidGreen2-based staining conditions

The incubation period between a fluorescent dye and a sample has a strong influence on the fluorescence-based quantification and reproducibility of the methodology and is therefore an important variable to be examined. In order to understand the consequence of the incubation time on the staining with LipidGreen2, long-term measurements were carried out. After reaching the maximum and stable fluorescence intensity at 200 min, a slight decrease was observed as a function of time (Fig. 2a). Nevertheless, an incubation of 200 min is not suitable for a fast online monitoring approach. Since the staining follows an approximate logarithmic curve, a linear relationship can be assumed for the first 100 min of incubation. These findings could be confirmed in subsequent experiments, where the intensity was monitored at three different light intensities over a period of 120 min (Fig. 2b). A robust linear relationship up to the first 40 min of incubation was determined under ambient (R^2 0.9986) and under light exclusion (R^2 0.9965) conditions. After 40 min, the slope of the signal at ambient conditions decreased. Hence, it can be concluded that the influence of light can no longer be neglected. Incubation at high intensity lighting (100 μmol m^{-1} s^{-1}) showed high photobleaching effects at the early stage of the incubation process, and therefore cannot be used for LipidGreen2-based fluorescence quantification.

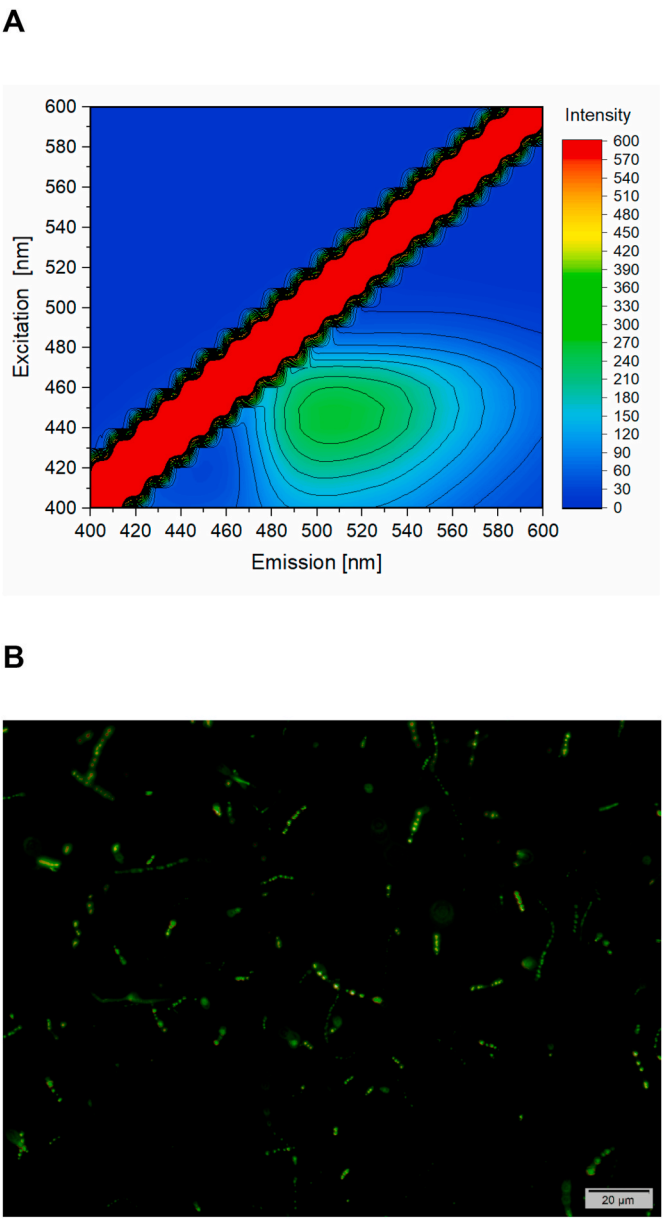


Fig. 1. (A) 3DEEM of PHB producing *C. necator*. **(B)** Bioimaging fluorescent PHB in *C. necator*. Incubation took place under light exclusion for 30 min with 2.6 μM LipidGreen2.

In consideration of these findings, an incubation time of 10 min under ambient lighting was set for subsequent PHB quantification experiments.

3.3. LipidGreen2-based monitoring of PHB production

3.3.1. Correlation between side scatter and biomass concentration

The cell concentration has a direct impact on the fluorescence and can negatively influence the PHB emission signal. The so-called inner filter effects (IFE) affect scattering, diffraction, and reflection of both excitation and emission signals [29]. These effects increase as the biomass concentration increases. To reduce this influence, a consistent optical density should be applied to all samples. However, this adjustment increases the potential for errors, reduces reproducibility, and represents an additional manual step [30]. Furthermore, not all fluorescence spectrometers are suitable for optical density measurements, whereby a second device is needed. Hence, we developed a different

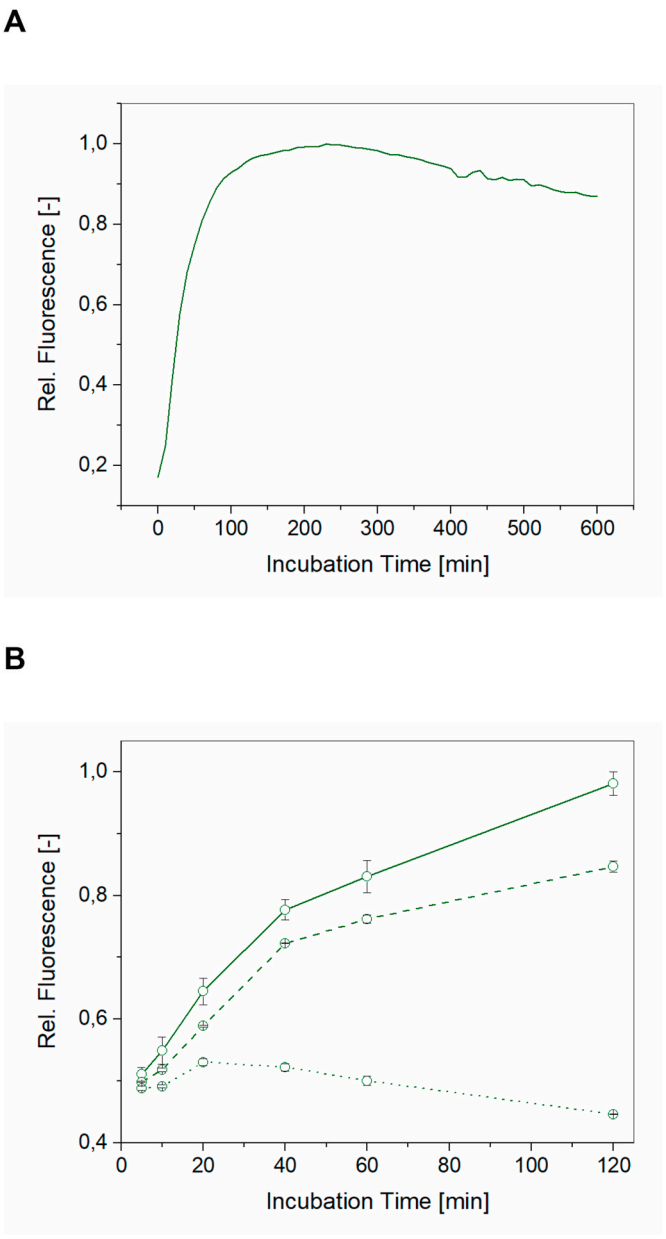


Fig. 2. **(A)** Long-term stability measurement of LipidGreen2 staining PHB in *Cupriavidus necator*. **(B)** Effect of different light intensities on the fluorescence of *C. necator* as a function of the incubation time. Regular line: 0 μmol $m^{-2}s^{-1}$, dashed line: 10 μmol $m^{-2}s^{-1}$, dotted line: 100 μmol $m^{-2}s^{-1}$. The excitation and emission wavelengths were set to 440 nm and 505 nm respectively.

approach by using constant cell culture to buffer ratio regardless of the biomass concentration. Before the fluorophore was added and the fluorescence itself was measured, the side scatter (SSC) of the sample was recorded using the same spectroscopic parameter constellation on the same device as for the fluorescence measurements. By plotting biomass against SSC, a robust linear correlation up to a biomass concentration of 6.6 g L^{-1} was identified (Fig. 3a). This proves that both the scattered light and, therefore, the IFE were proportional to the biomass used. Comparative approaches were also successful in flow cytometry, where the SSC was used as a measure of the granularity of cells and the number of vesicles within a cell [31].

3.3.2. *Quantification of PHB and correction of inner filter effects*

In order to use the fluorescence signal to quantify intracellularly accumulated PHB, two different approaches can be followed. Firstly, the

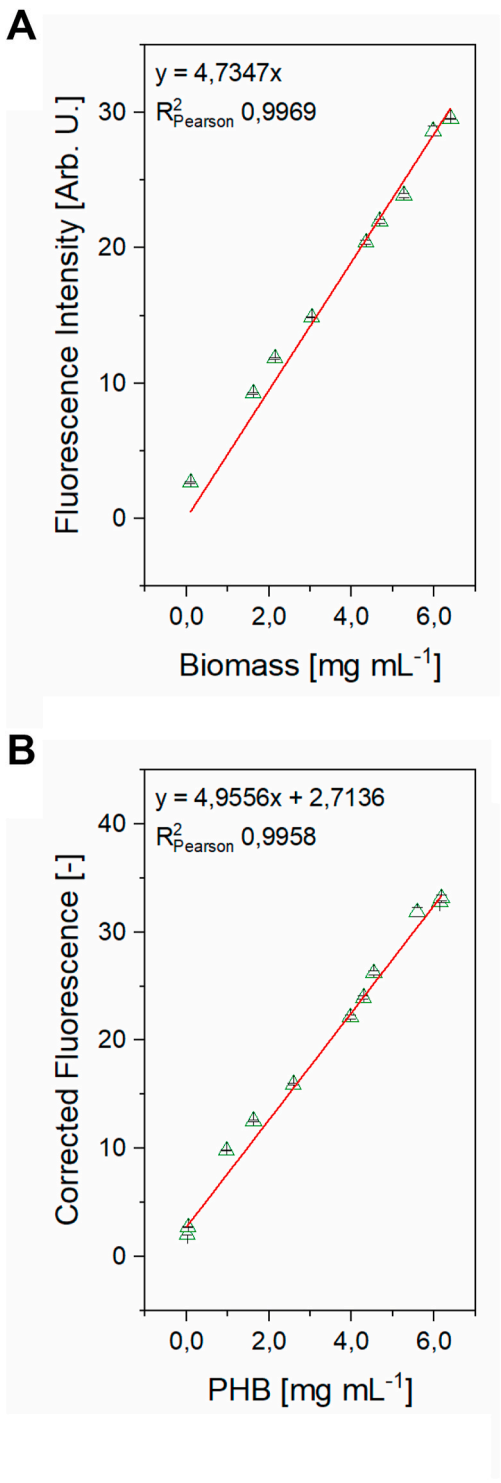


Fig. 3. Linear correlation between **(A)** biomass concentration and sample side scatter and **(B)** PHB content determined by HPLC and corrected fluorescence intensities of LipidGreen2. Excitation and emission wavelengths were 440 nm and 505 nm, respectively, for all measurements.

fluorescence signal can be compared with the PHB content using external methods such as gravimetry or HPLC. Secondly, a PHB standard curve can be created directly at the fluorescence spectrometer using a solid PHB standard of analytical grade. The latter approach was unsuccessful in our experiments. This can be explained by the insolubility of PHB powder in aqueous solution, whereby a suspension must be used. Due to the granular packing, the suspension has a different

microenvironment than the native amorphous PHB [32]. As a result of these differences, the number of fluorophore molecules that accumulate inside the hydrophobic PHB can differ significantly between the two states and consequently result in different fluorescence intensities and maximum emission wavelength.

For this reason, we used a well described HPLC approach for the external quantification of PHB and identified a moderate agreement (R^2 0.9206, data not shown) between the raw fluorescence intensities and the amount of PHB measured by HPLC. To improve the correlation, the SSC was incorporated to correct the IFE. We perceived a high reciprocal relationship between the sum of SSC and LG2 fluorescence that can be described as:

$$\frac{1}{[PHB]} = \sum_{i=1}^{n} \frac{1}{Int_i} \quad (1)$$

where Int represents the signal intensity of either the SSC or the fluorescence intensity at 505 nm and [PHB] the concentration of PHB analyzed by HPLC. By transforming equation (1), the PHB content can be calculated directly using the raw data of LG2 fluorescence and the corresponding SSC value as follows:

$$[PHB] = \frac{Int_{SSC} \cdot Int_{LG2}}{Int_{SSC} + Int_{LG2}} \quad (2)$$

Using this equation, the coefficient of determination was significantly improved to 0.9912 (Fig. 3b), which also compares more accurately with LipidGreen1 (R^2 0.96) [23]. The methodology could be verified over the entire cultivation and covered a range of up to 6.1 g L^{-1} and made it possible to obtain analytical PHB contents. The assay showed only limitations at insufficient concentrations, making this method optimal as a fluorescence-based online tool for determining optimal harvest points (Fig. 4). Furthermore, this method can be developed into a rapid kit to determine the presence and quantity of PHB in a sample in less than 15 min.

4. Conclusion

Fluorescence measurements offer an excellent alternative for the rapid determination of PHB during fermentation. Since fluorescence staining is a delicate process, many parameters need to be evaluated to obtain a reliable and reproducible assay. While Nile Red is one of the most commonly used and studied fluorescent dyes, it often lacks reproducibility of the assays. LipidGreen2, a small, sensitive, and versatile probe with a high hydrophobicity index, has recently been reported to stain neutral lipid selectively. We demonstrated quantification and bioimaging of PHB based on LipidGreen2 fluorescence for the first time, using sample side scatter to correct the inner filter effects. This novel approach enables different biomass concentrations to be used and avoids the need for different devices. The strong linearity obtained allows an exact PHB quantification in just 15 min, following a comprehensible and simple protocol. This study also highlighted the successful identification of suitable excitation and emission wavelengths by 3DEEM as well as fluorophore stability determinations of the probe, demonstrating optimal characteristics of LipidGreen2 for the detection of lipophilic storage compounds. In addition, limitations, such as low sensitivity or high background signal observed when using Nile Red or BODIPY, are lacking. Based on these investigations, we could determine that LipidGreen2 is a promising, easy to handle fluorescent marker for the selective determination of storage lipids in microorganisms. Further studies will focus on LipidGreen2-based PHB quantification in photoautotrophic microorganisms such as microalgae since emission of their primary fluorophores, e.g. chlorophyll or phycobilins [33], does not interfere with LipidGreen2.

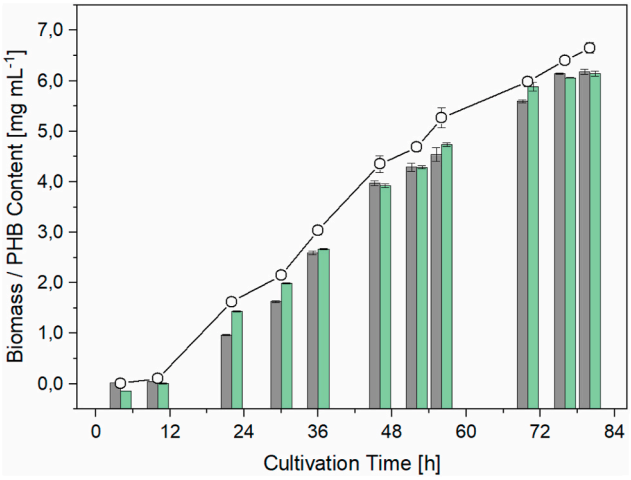


Fig. 4. Comparison of PHB-content determined by LipidGreen2 assay (green bars), by HPLC (gray bars) and biomass content (dotted line) over a cultivation time of 79 h. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Credit author statement

Alexander Kettner: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Data curation, Writing - original draft. Carola Griehl: Supervision, Writing - review & editing, Resources, Funding acquisition. Herewith I ensure, that all authors agreed to the descriptions and the final draft; and that no changes in the manuscript or other related documents were made afterward.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2020.100819.

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