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Short Communication

An environmentally-friendly fluorescent method for quantification of lipid contents in yeast

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HIGHLIGHTS

- Nile red staining offers short analysis time for neutral lipid determination in yeast.
- Nile red staining is energy efficient and uses minute volumes of organic solvents.
- Nile red staining is suitable for fast screening and high-throughput analysis.

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ABSTRACT

This study aimed at developing an efficient, fast and environmentally-friendly method to quantify neutral lipid contents in yeast. After optimising the fluorescence instrument parameters and influence of organic solvent concentrations, a new method to quantify neutral lipids in yeast based on fluorescence was demonstrated. Isopropanol and Nile red in concentrations of 5% (final volume%) and 500 μg/L, respectively, were added to washed cells suspended in potassium chloride phosphate buffered saline (PBSKCl). Fluorescence was measured after 10 min in the dark. Glyceryltrioleate was used as model lipid and the calibration curve showed linearity (R^2 = 0.994) between 0.50 and 25 mg/L. Compared with traditional gravimetric analysis, the developed method is much faster and uses less organic solvents. Lipid contents determined by fluorescence and gravimetry were the same for some strains, but for other strains the lipid contents determined by fluorescence were less. This new method will therefore be suitable for fast screening purposes.

1. Introduction

The use of biofuels as a renewable, environmentally-friendly alternative to fossil fuels has been subject of research for many years. The most common biofuel in Europe is biodiesel. The global biodiesel market is estimated to reach 37 billion gallons by 2016 with an average annual growth of above 40% (Li et al., 2008). Nevertheless, traditional 1st generation (1G) biodiesel production (transesterification of triacylglycerols from plant oils) has various drawbacks and limitations (Kalscheuer et al., 2006) such as: season and climate-dependent cultivation of the plant oil feedstock (rapeseed, soybean); the agricultural land competition for food, resulting in reduction of cultivated area for feed and consequently increasing food prices; and international pressure to reduce the use of terrestrial plants in biofuels production. It is therefore of ultimate importance to evaluate other lipid sources in addition to plants. Possible alternative oil sources are oleaginous microorganisms, such as microalgae, yeasts and fungi. Fermentation employing oleaginous microorganisms (Amaretti et al., 2012; Papanikolaou, 2011) (2G biodiesel production) is a very promising alternative solution to overcome the critical bottlenecks of 1G biodiesel production.

In comparison to other oleaginous fungi, the oleaginous yeasts are much more robust and easy to handle in the laboratory and large scale applications (Papanikolaou, 2011). Traditional methods for measurement of lipid content of microbial biomasses are based on solvent extractions followed by gravimetric determination of either total, polar or neutral lipids (e.g. Poli et al., 2013; Sitepu et al., 2012). There are two major drawbacks of the conventional method: (1) the results are quite dependent on the cell wall lysis step (Poli et al., 2013; Sitepu et al., 2012) hence highly time and labour consuming, infeasible for screening; (2) the method uses non-environmental friendly and strong organic solvents such as

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chloroform ($CHCl_3$) that should be handled with care. Therefore, there is a need for a rapid, robust and highly efficient method for quantifying lipid contents in microbial biomasses. Particular attention has recently been paid to a fluorescence-based method to evaluate lipid levels in different cells using the fluorescent dye Nile red. Kimura et al. (2004) published a method to determine lipid contents of yeast cells applying Nile red to yeast cells re-suspended in potassium phosphate buffer (PBS). Quite recently Sitepu et al. (2012) evaluated the method by Kimura et al. (2004) and suggested addition of 5% dimethylsulfoxide (DMSO) for improving the penetration of Nile red into the yeast cells. However, neither of the methods could adequately quantify the lipids, which is essential for allowing screening and evaluation of oleaginous yeasts for their ability to produce lipids. The aim of this study was therefore to develop a highly efficient and fast method to quantify neutral lipid contents in yeasts using less aggressive organic solvents and to investigate the factors affecting the performance.

2. Methods

2.1. Microorganism and culture conditions

The yeast strain *Yarrowia lipolytica* QU21 (Poli et al., 2013) and three yeast isolates (YI1, YI2 and YI3) obtained from a biodiesel plant (Emmelev A/S, Denmark), were used for evaluation of the method and were pre-grown on YEPD or YEPG agar (1% yeast extract, 2% peptone, 2% glucose or glycerol, 2% agar). The liquid medium for cell culture growth was composed of: 10% glucose or glycerol, 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, and 0.05% $MgCl_2{\cdot}6H_2O$. Glucose was used for *Y. lipolytica* and glycerol was used for YI1, YI2 and YI3. All microorganisms were pre-cultured for 24 h, followed by inoculation in 100 mL of the corresponding liquid media using 250 mL Erlenmeyer flasks. The submerged cultures were incubated in a rotating shaker at 150 rpm and 25 °C for 5 days. Liquid cultures were centrifuged (Heraus Multifuge X3R Centrifuge, Thermo Scientific, AXEB, Denmark) at 4700*g* for 10 min to remove the supernatant, and used for dry biomass determination, analyses of yeast lipids using the fluorescence method and gravimetric determination. The cell pellets were washed twice with 0.15 M potassium chloride (KCl) in 0.01 M phosphate buffered (pH 7.0) saline (PBSKCl) and, if necessary, re-suspended in PBSKCl.

2.2. Dry biomass determination

Cell pellets (described in Section 2.1) were kept at 70 °C to constant weight.

2.3. Fluorescence spectroscopy

2.3.1. General procedure

Samples were diluted to 10 mL with PBSKCl containing 5% (volume%) isopropanol (i-PrOH), unless otherwise stated. Fluorescence in arbitrary units (A.U.) was measured using a Cary Eclipse fluorescence spectrophotometer controlled by the Cary Eclipse software version 1.1.1.3 (Varian, Analytical Instruments, Værløse, Denmark) before and after addition of 50 μL Nile red, in order to subtract the intrinsic fluorescence of the sample. Samples added Nile red remained 10 min in the dark before analysis. To measure the neutral lipids, excitation and emission wavelengths of 488 and 585 nm, respectively, were used (Kimura et al., 2004). A 10 mg/L solution of the standard lipid resulted in a fluorescence intensity in A.U. of about 23. 100 mg/L Nile red was prepared in acetone (DMK).

2.3.2. Determining the optimal model lipid as standard

Three different model lipids, glyceryltripalmitate, $C16{:}0_3$ (GTP), glyceryltrioleate, $C18:1_3^{\Delta 9}$ (GTO) and glyceryltrilinoleate, $C18:1_3^{\Delta 9,12}$ (GTL), where the acyl-groups represent different degrees of saturation, were selected to determine the possibility to differentiate among the degree of saturation in the fluorescence assay, and thereby determine the lipid saturation profile of the yeast strains. Stock solutions of GTP, GTO and GTL were prepared in i-PrOH. Working solutions were prepared by diluting the stock solutions 10 times in PBSKCl, reaching the final concentration of 5, 15 and 1.5 mg/L, respectively.

2.3.3. Influence of organic solvents and yeast cell quantities

A range of organic solvents (ethanol (EtOH), i-PrOH, DMK, DMSO and combinations thereof) were assessed for their ability to enhance/suppress the Nile red penetration into the cell and compared with water as well as their influence on the fluorescence signal of the Nile red–lipid complex. According to the general procedure, the fluorescence of a mixture of growth medium:organic solvent in the ratio 1:4 was measured before and after addition of Nile red.

An experiment using increasing quantities of yeast cells and different organic solvents was set up. The aim was to study the influence of yeast cell quantity on the lipid determination and if the type of organic solvent affected the Nile red penetration through the cell membrane. Linear regression was used to evaluate the potential influence of quantities of yeast cells and organic solvent on the lipid determination. 10–200 μL volumes (corresponding to 2.77–55.3 mg/L according to the cell dry weight) were diluted to 10 mL with PBSKCl with or without 10% (volume%) i-PrOH or DMSO. The fluorescence of the samples was measured as described in Section 2.3.1.

2.3.4. Optimising i-PrOH concentration, mixing intensity and fluorescence signal

An experimental design using Plackett and Burman with 12 assays (PB12) and 3 centre points run was performed to evaluate i-PrOH concentration, mixing intensity and fluorescence signal. The i-PrOH concentration was tested at 5%, 10% and 20% (volume%) in the analysis solution. Vortexing for 1 min, gentle manual mixing three times were compared with no mixing at all to find the optimal mixing intensity. The fluorescence signal is influenced by when the signal is recorded, how long the instrument scans the emitted light, at which voltage the photomultiplier tube (PMT voltage) is operated and the slit width. The signal was recorded immediately, after 30 s and after 180 s. The instrument scan times were studied at 0.1; 3 and 10 s. PMT voltages of 400, 600 and 800 V and slit widths of 2.5; 5 and 10 nm were tested.

2.4. Lipids extraction

An equivalent of 0.14 g dry biomass was re-suspended in 10 mL PBSKCl, kept on ice while sonicated for 10 min using Branson Sonifier Cell Disruptor S-250A (Branson Ultrasonics Corporation, VWR Bie & Berntsen A/S, Herlev, Denmark), and centrifuged at 4700*g* for 10 min at 20 °C. The lipids were extracted from the disrupted cells using a $CHCl_3$:MeOH solution (2:1) according to Folch et al. (1957), the organic phase was isolated and evaporated to constant weight.

2.5. Calibration curves and statistical analysis

Linear regression of GTO (0.50–25 mg/L) was used to quantify yeast cell lipid contents using the Nile red fluorescence method. The experimental design analysis (PB12) was carried out using Statistic 5.0 software (Statsoft, USA). The lipid content mean values ($n = 3$) are reported and compared by analysis of variance

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