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Rapid quantification of microalgal lipids in aqueous medium by a simple colorimetric method

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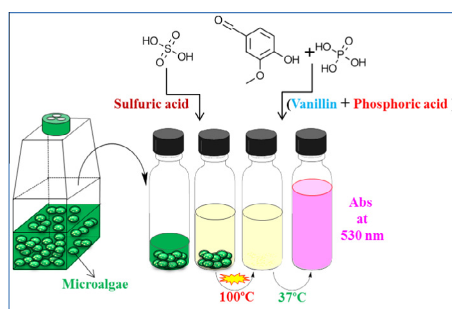
HIGHLIGHTS

- A rapid colorimetric method for lipids quantification of microalgae.
- Rapid and accurate lipid quantification within four different microalgae.
- Verification of SPV's accuracy via comparison with gas chromatography.

GRAPHICAL ABSTRACT



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ABSTRACT

Identification of novel microalgal strains with high lipid productivity is one of the most important research topics in renewable biofuel research. However, the major bottleneck in the strain screening process is that currently known methods for the estimation of microalgal lipid are laborious and time-consuming. The present study successfully employed sulpho-phospho-vanillin (SPV) colorimetric method for direct quantitative measurement of lipids within liquid microalgal culture. The SPV reacts with lipids to produce a distinct pink color, and its intensity can be quantified using spectrophotometric methods by measuring absorbance at 530 nm. This method was employed for a rapid quantification of intracellular lipid contents within *Chlorella* sp., *Monoraphidium* sp., *Ettlia* sp. and *Nannochloropsis* sp., all of which were found to have lipid contents ranging in between 10% and 30%. Subsequent analysis of the biomass using gas chromatography confirmed that our protocol is highly accurate (R^2 = 0.99).

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1. Introduction

Dwindling global fossil fuel reserves combined with perpetually increasing demands have influenced many governments and organizations across the world to seek suitable alternative source of transportation fuels, such as biodiesel to replace current petroleum based fuels (Knothe et al., 1997; Pulz and Gross, 2004). One particular candidate as feedstock for sustainable biodiesel production is microalgal biomass, which has several distinct advantages such as sustainability and high productivity over conventional feedstock

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such as oil crops, animal fat, and waste oils (Chisti, 2007). There are many algae that are capable of altering their lipid biosynthetic pathways towards the formation and accumulation of neutral lipids (20–50% DCW) in the form of triacylglycerides, which can be converted into biodiesel by transesterification reaction (Franz et al., 2013; Hu et al., 2008). However, the primary barrier to the commercialization of microalgal biodiesel is the high costs associated with the biomass production (Brennan and Owende, 2010). This problem can be alleviated not only by development of new downstream processes, but also by searching for appropriate strains of microalgae with favorable characteristics such as high lipid productivity (Demirbas and Fatih Demirbas, 2011).

The primary reason behind the slow progress in strain development is due to the lack of rapid, high throughput lipid quantification technologies. Currently, most widely used screening methods for microalgal lipid productivity include gravimetric methods, gas chromatography, and staining with lipophilic fluorescent dye for quantitative spectrophotometry (Bligh and Dyer, 1959; Chen et al., 2009). The gravimetric method for lipid content determination comprises of solvent assisted lipid extraction, usually involving organic or chlorinated solvents such as hexane or chloroform, of a known quantity of dried biomass. Exact weight of the extracted lipid is taken after the evaporation of extracting solvent in order to obtain percentage of intracellular lipid. Major downside of gravimetric method is that a relatively large quantity of sample is necessary; in addition it requires time consuming drying and extraction processes. The Bligh and Dyer method can be used for transesterification of all intracellular lipids, and resulting fatty acid methyl esters (FAME) could be analyzed using various analytical method such as gas chromatography with mass spectrometry (GC/MS) (Bigelow et al., 2011; Blokker et al., 2002) or high pressure liquid chromatography (HPLC) (Jones et al., 2012). Such analytical methods require less quantity of sample compared to gravimetric methods for accurate quantification and also can provide data on precise lipid composition. However, labor intensive sample preparation steps are required, and care must be taken in order to make sure complete conversion of the lipid occurs in addition to preventing oxidative degradation of the samples. Also this process extracts chlorophyll in addition to neutral lipids, which can result in overestimation of total lipid within the biomass which is appropriate for use as biofuels (Archanaa et al., 2012).

Due to the fact that the drying and extraction processes are time consuming and labor intensive, some have attempted to develop more rapid quantification methods using lipophilic fluorescent dyes with Nile Red or BODIPY (Bertozzini et al., 2011; Cooksey et al., 1987). Due to the fact that microalgal cells possess thick cell wall that prevents penetration of dye into the intracellular matrix, carrier solvents such as DMSO have been used to assist with the staining process (Chen et al., 2011; Cooper et al., 2010; Velmurugan et al., 2013). However, broad spectrum lipid quantification assay is not possible using lipophilic dyes, since correlation between fluorescence intensity and actual lipid content can vary among different strains (Cooper et al., 2010). This primarily is due to the fact that the strains with greater cell wall thickness have lower staining efficiency. Therefore, fluorescent dye method remains a qualitative analysis at best and is not an effective method for lipid quantification.

Sulfo-phospho-vanillin (SPV) reaction was first introduced by Chabrol and Charonnat in 1937 and was used as a standard routine for estimation of total lipids in human cerebrospinal fluid (Vatassery et al., 1981). An improved version of the SPV reaction was introduced; including a colorimetric method for the quantification of total lipid within a sample was developed by Drevon and Schmit (1964). As of today, the sulpho-phospho-vanillin reaction is a widely utilized tool in medical field that is capable of accurate and rapid quantification of lipids within human serum. In brief this method involves: (a) preparation of phospho-vanillin reagent using phosphoric acid and vanillin, (b) addition of concentrated sulfuric acid to a sample that contains unsaturated lipids of interest, and heating of the mixture, (c) then followed by the addition of phospho-vanillin reagent, (d) measurement of absorbance at 530 nm (Knight et al., 1972). The application of sulpho-phospho-vanillin reaction for rapid screening of wild type and mutant microalgal strain would be a significant step forward from the time consuming or imprecise past techniques. The proposed method requires small amount of biomass in the orders of milligrams or less, does not require drying and extraction step, and much simpler and rapid compared to any other existing techniques.

2. Methods

2.1. Chemical and reagents

All the solvents and chemicals used were purchased from Sigma–Aldrich, unless specified otherwise.

2.2. Cultures and growth conditions

Ettlia sp., *Monoraphidium* sp., *Chlorella vulgaris* and *Nannochloropsis oceanica* were selected as the model microalgae for the present study. From these strains, only *Nannochloropsis oceanica* is a marine strain and rests of others are fresh water strains. *Ettlia* sp. was obtained from Korea Research Institute of Biology and Biosciences (KRIBB), Daejeon in South Korea, and *Monoraphidium* sp. was isolated in the Bio-Energy Engineering Research Lab of KAIST (unpublished result). The fresh water algae were cultured in BG11 and Marine *Nannochloropsis oceanica* strain was mass cultivated in outdoor raceway pond.

2.3. Standard curves preparation of lipid standard solutions

The standard lipid stocks were prepared using commercial canola oil at 20 mg in 10 ml chloroform (final concentration, 2 mg/ml), which was subsequently stored at −20 °C before use. Different amount of lipid in microliters of standard oil solution was added in the empty tube. The tubes were kept at 60 °C for 10 min to evaporate the solvent and 100 μl of water was added to the lipid standard. Further sample was prepared by following SPV reaction methods. Teflon-covered glass vials were used throughout all experiments.

2.4. Proposed sulfo-phospho-vanillin assay for lipid estimation

Phosphovanillin reagent was prepared by initially dissolving 0.6 g vanillin in 10 ml absolute ethanol; 90 ml deionized water and stirred continuously. Subsequently 400 ml of concentrated phosphoric acid was added to the mixture, and the resulting reagent was stored in the dark until use. To ensure high activity, fresh phospho-vanillin reagent was prepared shortly before every experiment run. For SPV reaction of the algal culture for lipid quantification, a known amount of biomass in 100 μl water, which are either suspended in a known volume of liquid culture or harvested via centrifugation at 4000 RPM for 5 min, was used. 2 mL of concentrated (98%) sulfuric acid was added to the sample and was heated for 10 min at 100 °C, and was cooled for 5 min in ice bath. 5 mL of freshly prepared phospho-vanillin reagent was then added, and the sample was incubated for 15 min at 37 °C incubator shaker at 200 rpm. Absorbance reading at 530 nm was taken in order to quantify the lipid within the sample.

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