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Simple, fast and accurate method for the determination of glycogen in the model unicellular cyanobacterium *Synechocystis* sp. PCC 6803

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ABSTRACT

Glycogen is a highly soluble branched polymer composed of glucose monomers linked by glycosidic bonds that represents, together with starch, one of the main energy storage compounds in living organisms. While starch is present in plant cells, glycogen is present in bacteria, protozoa, fungi and animal cells. Due to its essential function, it has been the subject of intense research for almost two centuries. Different procedures for the isolation and quantification of glycogen, according to the origin of the sample and/or the purpose of the study, have been reported in the literature. The objective of this study is to optimize the methodology for the determination of glycogen in cyanobacteria, as the interest in cyanobacterial glycogen has increased in recent years due to the biotechnological application of these microorganisms. In the present work, the methodology reported for the quantification of glycogen in cyanobacteria has been reviewed and an extensive empirical analysis has been performed showing how this methodology can be optimized significantly to reduce time and improve reliability and reproducibility. Based on these results, a simple and fast protocol for quantification of glycogen in the model unicellular cyanobacterium *Synechocystis* sp. PCC 6803 is presented, which could also be successfully adapted to other cyanobacteria.

1. Introduction

Cyanobacteria are ancestors of chloroplasts and share common and conserved photosynthetic processes and metabolic pathways, including carbon storage. Carbon fixed in the photosynthetic dark reactions is stored in the form of starch in chloroplasts of eukaryotic photosynthetic organisms or glycogen in cyanobacteria (Ball et al., 1996; Cenci et al., 2014). Glucose is linked through the α(1 → 4) and α(1 → 6) glycosidic bonds. The α(1 → 4) glycosidic bonds yield a linear polymer called amylose, whereas the α(1 → 6) glycosidic bonds yield a branched polymer called amylopectin (Fig. 1A). Glycogen and starch are both polymers of glucose, but they are structurally different and show different physicochemical properties, due to the different distribution of the α(1 → 4) and α(1 → 6) glycosidic bonds in the molecule. The glycogen is a highly branched amylopectin (Fig. 1B), while the starch is composed of amylose and amylopectin at different proportions. The average amylose and amylopectin content of the starch is 20–30% and 70–80%, respectively. The starch accumulates in chloroplast of plant cells and microalgae in the form of water-insoluble semi crystalline granules (Buléon et al., 1998), whereas the glycogen accumulates as water-soluble granules in the cytosol of bacteria, protozoa, fungi and animal cells. The structure of glycogen is similar to amylopectin, with a substantially higher branching density. It consists of 90–93% of α-D-(1–4) glycosidic bonds and 7–10% of α-D-(1–6) glycosidic linkages (Calder, 1991). In general, there is approximately one branch point per 10–14 residues in glycogen and 20–25 in amylopectin. The links between chains are randomly organized in the glycogen molecule and different structure models have been proposed based on both theoretical and empirical analysis (Manners, 1991). The empirical evidences that use the enzymatic analysis indicate that the glucose residues in the glycogen molecule are organized resulting in a structure similar to a tree or a grape, also called "Whelan-structure" (Gunja-Smith et al., 1970). More recent research corroborates that glycogen is a hyperbranched macromolecule with broad size distribution (Fernandez et al., 2011). The high level of branching results in an amorphous and compact structure and a high aqueous solubility that ensures rapid mobilization of energy through glycogen degrading enzymes (Meléndez et al., 1998).

Abbreviations: AMG, amyloglucosidase; DNS, Dinitrosalicylic Acid Reagent; GO, glucose oxidase; GOD, LabAssay Glucose method; GOPXOD, glucose oxidase-peroxidase method; HK-G6PD, hexokinase-glucose-6-phosphate dehydrogenase method; *O*D, *o*-dianisidine; PX, peroxidase

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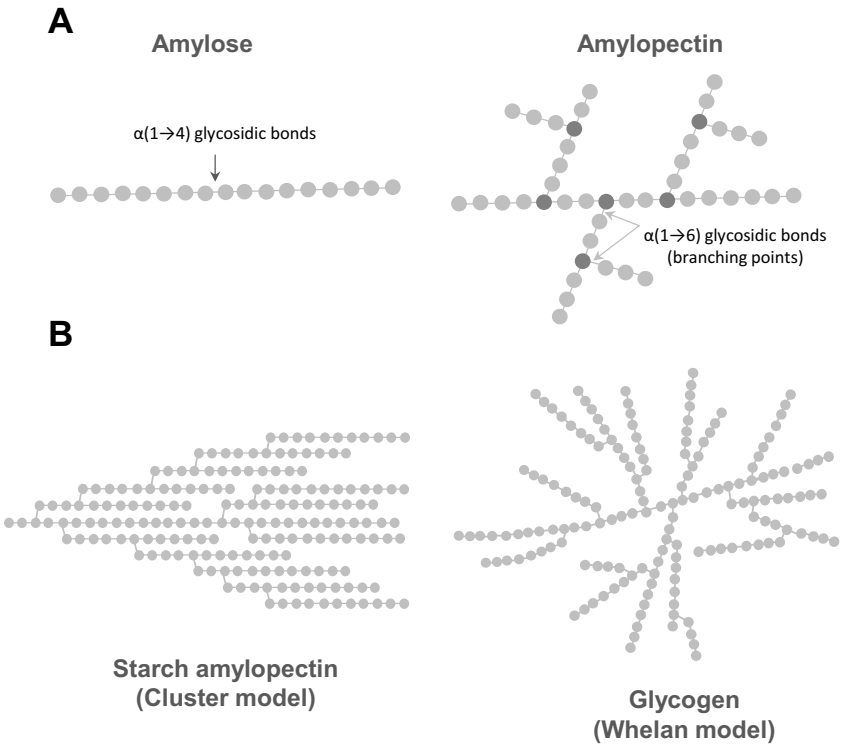

Fig. 1. Molecular composition of glycogen and starch. A) In starch, the branching points of amylopectin are arranged in clusters not randomly distributed along the macromolecule, which contributes to the crystallinity of the starch granule. B) In glycogen, the branching points are organized randomly (Whelan model), resulting in a highly compact and hydrophilic structure of the glycogen granule.

Glycogen is observed under the electron microscope as a particle of 60–200 nm in diameter, called α-particle. The α-particle represents the highest level of glycogen organization and appears as a rosette-like structure composed of small spherical units, called β-particles. Each β-particle corresponds to a single Whelan-type branched glycogen particle (Calder, 1991). β-particles are held together covalently in the α-particle (Sullivan et al., 2010).

The increasing demand for renewable energy sources and chemicals has raised the interest on photoautotrophic cyanobacteria as feedstock. Certain strains of cyanobacteria have been engineered to produce a variety of valuable products, such as ethanol, succinate, n-butanol, fatty acids, sucrose, acetone or isoprene, among others (Zhou et al., 2012; Jacobsen and Frigaard, 2014; Kudoh et al., 2014; Anfelt et al., 2015; Hasunuma et al., 2018; 2016). However, the production rate in general is low, hindering the economic viability of the process. The metabolic pathways for the synthesis of storage compounds have been investigated in these microorganisms, as these compounds can be a target for metabolic strategies in order to increase the productivity of other valuable substances. Metabolic engineering is one of the most promising tools to increase the productivity of cyanobacteria as microbial cell factories, being glycogen one of the key targets proposed in metabolic engineering approaches. Increase in the production of ethanol (Namakoshi et al., 2016), lactate (van der Woude et al., 2014) or PHB (Wu et al., 2002) has been achieved by inactivation of the pathway for glycogen synthesis in genetically engineered cyanobacteria. Moreover, although glycogen is relatively less examined as feedstock for the production of biofuels, it has the potential to become a promising alternative to fossil resources. The synthesis of glycogen from CO_2 by photosynthesis and its subsequent hydrolysis makes glycogen an abundant and renewable feedstock for the production of glucose, which could be converted into bioethanol in a subsequent fermentation reaction (Klein et al., 2015). It has also physicochemical properties whith strong potential in pharmaceutical industry (Gopinath et al., 2018).

Taking into account the potential of glycogen in future applications of cyanobacteria, it is necessary to have an accurate and standardized method for its quantification. Glycogen was firstly isolated from liver tissue (Young and Claude, 1957) due to its physiological importance in human health, and several methods have been developed for more than one century towards its quantification. The most widespread method for the quantification of glycogen was firstly developed by Pflüger in 1902 for liver glycogen (Pflüger, 1902). The Pfluger's method involves the alkaline hydrolysis of tissues for the extraction and solubilisation of glycogen and the subsequent recovery of glycogen by precipitation with cold ethanol. Once the glycogen is recovered, a procedure for quantification is applied. A variety of methods for the quantification of glycogen have been reported so far for many different cell types. For cyanobacteria, reported procedures for quantification of glycogen are also diverse. In the present work, an exhaustive review of the methodology for the quantification of glycogen in cyanobacteria is presented, together with experimental data showing the optimization of the protocol. The most accurate and precise procedure is presented with the aim to achieve the standardization of the methodology for quantification of cyanobacterial glycogen.

2. Material and methods

2.1. Strains and culture conditions

Glucose-tolerant (GT) strain of *Synechocystis* sp. PCC 6803 was grown photoautotrophically in BG-11 medium supplemented with 12 mM NaHCO3 at 30 °C, under continuous illumination in an orbital shaker. Cells were harvested by centrifugation and pellets used for glycogen determinations.

2.2. Extraction of glycogen

Glycogen was extracted from wet cell pellets and comparison of different extraction techniques was performed.

2.2.1. Mechanical cell disruption

Extraction of glycogen using mechanical disruption of cells adapted from a previous report (Yoo et al., 2015) was applied as follows: 0.5 mg of biomass was suspended in 200 µl of distilled water and 100 µl of glass beads (0.25–0.3 mm, Sigma-Aldrich, St. Louis, MO, United States) was

added. Samples were subjected to five cycles (20 s: 20 s) of agitation: incubation on ice, using a Mini-beadbeater (Mini-Bead-Beater-16, Biospec Products). After cell disruption, samples were centrifuged at 13.000 rpm for 15 min and the supernatant was incubated at 100 °C for 15 min and used for glycogen quantification.

2.2.2. Alkaline hydrolysis

Extraction of glycogen by alkaline hydrolysis adapted from a previous work (Ernst et al., 1984) was applied as follows: 200 µl of KOH 30% was added to 50 µl of cells concentrated in distilled water and boiled at 100 °C for 1.5 h. After extraction, 0.8 ml of cold ethanol was added and incubated 30 min on ice to allow precipitation of glycogen. The precipitate was recovered by centrifugation at 4 °C, 13000 rpm for 15 min. Pellet was washed twice with ethanol, dried, suspended in 200 µl of 2.5 mM sodium acetate, pH 5.2 and used for glycogen quantification.

2.2.3. Thermolysis

For extraction of glycogen by thermolysis, methanol was added to samples, incubated overnight at 4 °C and centrifuged at 13000 rpm for 5 min. 250 µl of water was added to the dried cell pellet, boiled at 100 °C for 40 min and used for glycogen quantification.

2.3. Neutralization step

For conditions used in this work, 80 µl of glacial acetic acid 98% was added to 250 µl of samples previously subjected to alkaline hydrolysis, mixed and let it cool to room temperature before the glycogen quantification assay. Acetic acid volume was carefully selected to reach a pH value in the range 5.0–5.5 (Supplementary Table 1).

2.4. Specific quantification of glycogen

Samples containing glycogen were subjected to enzymatic hydrolysis using amyloglucosidase (AMG; A7095, Sigma-Aldrich) to obtain glucose monomers and subsequently glucose was quantified by the enzymatic colorimetric method of glucose oxidase (GO)-peroxidase (PX)-*o*-dianisidine (OD) (Huggett and Nixon, 1957). Two protocols were applied: “standard” and “in-house”.

Standard protocol: Glycogen digestion was performed by incubation of samples with 10 units of AMG at 55 °C for 2.5 h. Glucose was then quantified using the Glucose (GO) Assay Kit (GAGO-20, Sigma-Aldrich) following the manufacturer instructions: an assay reagent containing GO, PK and OD was added to samples and incubated at 37 °C for 30 min; after addition of H_2SO_4 (final concentration of 4.8 N), the absorbance was measured at 540 nm.

“In-house” protocol: a modification of the standard protocol based on the coupled activity of AMG, GO and PX, has been developed as follows: a reaction mixture containing 10 units of AMG, 5 units of GO (G7141, Sigma-Aldrich), 1 unit of PX (P8375, Sigma-Aldrich) and 0.05 mg of OD (D3252-o, Sigma-Aldrich) in 2.5 mM sodium acetate (pH 5.2) was added to 250 µl of the extracted samples (final volume of 1 ml), incubated for 30 min at 37 °C, and after addition of H_2SO_4 (final concentration of 4.8 N), the absorbance was measured at 540 nm.

As a control, replicates of each sample were performed without AMG treatment to quantify other substances that absorb at 540 nm and could interfere with the glycogen estimation. Absorption due to glycogen was calculated as follows: A_{540} (AMG-treated samples)-A_{540} (control samples). The resulting value was used to estimate glycogen concentration. Reference curves were performed using standard glycogen from oyster (G8751 Sigma-Aldrich) at the following concentrations: 0, 2.5, 5.0, 10.0, 15.0, 20.0 and 25.0 $\mu g \cdot ml^{-1}$ of glycogen.

3. Results and discussion

3.1. A comprehensive review of methodology for determination of glycogen in cyanobacteria

In cyanobacteria, glycogen was first identified by electron microscopy as a component of α-granules located among the photosynthetic thylakoids in the filamentous strain *Nostoc muscorum* (Chao and Bowen, 1971). Later different works aimed at describing the structure and functionality of glycogen in cyanobacteria were published (Weber and Wöber, 1975; Doolittle and Singer, 1974; Lehmann and Wöber, 1976; Fredrick, 1980; Yoo et al., 2002; Yoo et al., 2007; Hickman et al., 2013). As a result of these early reports, it was demonstrated its function to sustain growth under dark (Hanai et al., 2014), mixotrophic (Krishnakumar et al., 2015), photomixotrophic (Dong et al., 2016) and dark fermentation conditions (Aoyama et al., 1997; Carrieri et al., 2010), or in the acclimation to nitrogen starvation (Lehmann and Wöber, 1978; Kumar et al., 1983; Almon and Böger, 1988; De Philippis et al., 1992; Schneegurt et al., 1994; Schlebusch and Forchhammer, 2010) and salt stress (Fry et al., 1986; Page-Sharp et al., 1998). Thus, the literature about this subject is extensive and the procedures used for quantification of glycogen among different authors differ to a great extent. The estimation of glycogen content from tissues or cells involves a series of steps to allow extraction, solubilisation, purification and quantification. Procedures involved in the main steps of glycogen determination in cyanobacterial are reviewed and summarized in Table 1.

3.1.1. Methods for extraction of glycogen from cells

As shown in Table 1, a variety of methods for extraction of glycogen from cells has been reported, which can be classified into four types: i) alkaline hydrolysis, ii) acid hydrolysis, iii) mechanical disruption of cells and iv) thermolysis. Alkaline hydrolysis consists of the incubation of cells with strong alkali at high temperature to obtain a cell hydrolysate which contains the soluble glycogen. Acid hydrolysis involves the incubation of cells with strong acid at high temperatures to obtain a cell hydrolysate retaining the glycogen digested into glucose. Mechanical disruption of cells compromises a variety of mechanical methods to break down cells and the recovery of the soluble fraction containing soluble glycogen. Thermolysis consists in the incubation of cells at 100 °C, previously extracted with methanol or ethanol and resuspended in distilled water.

Alkaline hydrolysis is the most used among researchers (more than half of total reports), while the others individually represent < 20% of total reports.

3.1.2. Methods for purification of glycogen from samples

The methodology to purify glycogen after the extraction step shows a high degree of diversity. Alkaline hydrolysis is followed by the precipitation of glycogen with cold ethanol, which ensures a high degree of recovery of glycogen and its purification of other cellular components. Acid hydrolysis is followed by direct quantification of glucose in the cell hydrolysates, as the acid promotes the complete hydrolysis of glycogen, preventing its purification from samples. On the other hand, mechanical cell disruption techniques are followed by the quantification of glycogen directly from supernatants or may include the recovery of glycogen by precipitation before the quantification step. Finally, thermolysis is usually followed by quantification of glycogen directly after boiling, without an intermediate purification step.

3.1.3. Hydrolysis of glycogen

The quantification step is also different between authors, which may include an intermediate step of digestion of glycogen into glucose (81% of reports) or perform the estimation of glycogen directly after extraction/purification (19%). The digestion of glycogen into glucose is performed by enzymatic treatment or acid hydrolysis. Enzymatic treatment consists in the incubation of the sample with AMG, which

Table 1
A review of methods for determination of glycogen content in cyanobacteria.

Extraction[a]	Precipitation[b]	Hydrolysis[c]	Determination[d]	Quantification[e]	Genus	Reference
Acid hydrolysis	–	Acid	GLUCOSE	DNS	*Synechocystis*	Dong et al., 2016
Acid hydrolysis	–	Acid	GLUCOSE	GOD	*Synechocystis*	Arisaka et al., 2019
Acid hydrolysis	–	Acid	GLUCOSE	GOPXOD	*Anabaena*	Kumar et al., 1983
Acid hydrolysis	–	Acid	GLUCOSE	GOD	*Synechocystis*	Iijima et al., 2016
Acid hydrolysis	–	Acid	GLUCOSE	GOPXOD	*Anacystis*	Doolittle and Singer, 1974
Acid hydrolysis	–	Acid	GLUCOSE	HPLC	*Synechocystis*	Kamravamanesh et al., 2018
Acid hydrolysis	–	Acid	GLUCOSE	HPLC	*Synechocystis*	Kamravamanesh et al., 2019
Acid hydrolysis		Acid	GLUCOSE	HPLC	*Synechocystis*	Troschl et al., 2018
Acid hydrolysis*	–	Acid*	GLUCOSE	HPLC	*Photosynthetic consortium*	Arias et al., 2018
Acid hydrolysis*	–	Acid*	GLUCOSE	HPLC	*Photosynthetic consortium*	Lanham et al., 2012
Acid hydrolysis	–	Acid	GLUCOSE	*O*-Toluidine	*Synechocystis*	Osanai et al., 2005
Acid hydrolysis	–	Acid	GLUCOSE	*O*-Toluidine	*Synechocystis*	Schlebusch and Forchhammer, 2010
Acid hydrolysis	–	Acid	GLUCOSE	*O*-toluidine	*Synechococcus*	Forchhammer and Tandeau de Marsac, 1995
Alkaline hydrolysis	YES	–	GLYCOGEN	Anthrone	*Anabaena*	Sarma and Kanta, 1979
Alkaline hydrolysis	YES	–	GLYCOGEN	Anthrone	*Anabaena*	Deb et al., 2019
Alkaline hydrolysis	YES	–	GLYCOGEN	Anthrone	*Cyanothece*	Schneegurt et al., 1994
Alkaline hydrolysis	YES	–	GLYCOGEN	Anthrone	*Microcystis*	Deb et al., 2019
Alkaline hydrolysis	YES	–	GLYCOGEN	Anthrone	*Spirulina*	Carrieri et al., 2010
Alkaline hydrolysis	YES	–	GLYCOGEN	Anthrone	*Synechocystis*	Pade et al., 2017
Alkaline hydrolysis	YES	–	GLYCOGEN	Anthrone	*Synechococcus*	Fry et al., 1986
Alkaline hydrolysis	YES	–	GLYCOGEN	HPLC	*Spirulina*	Aikawa et al., 2012
Alkaline hydrolysis	YES	–	GLYCOGEN	HPLC	*Spirulina*	Hasunuma et al., 2013
Alkaline hydrolysis	YES	–	GLYCOGEN	HPLC	*Spirulina*	Izumi et al., 2013
Alkaline hydrolysis	YES	–	GLYCOGEN	HPLC	*Spirulina*	Depraetere et al., 2015
Alkaline hydrolysis	YES	–	GLYCOGEN	HPLC	*Synechocystis*	Joseph et al., 2014
Alkaline hydrolysis	YES	–	GLYCOGEN	Anthrone	*Synechocystis*	Zhou et al., 2012
Alkaline hydrolysis	YES	Acid	GLUCOSE	GOPXOD	*Synechocystis*	de Marsac et al., 1980
Alkaline hydrolysis	YES	Acid*	GLUCOSE	GOPXOD	*Cyanothece*	Krishnakumar et al., 2015
Alkaline hydrolysis	YES	Acid	GLUCOSE	*O*-Toluidine	*Synechocystis*	Anfelt et al., 2015
Alkaline hydrolysis	YES	Enzymatic	GLUCOSE	Nelson	*Anabaena*	Morsy et al., 2019
Alkaline hydrolysis	YES	Enzymatic	GLUCOSE	Nelson	*Nostoc*	Morsy et al., 2019
Alkaline hydrolysis	YES	Enzymatic	GLUCOSE	GOPXOD	*Scytonema*	Page-Sharp et al., 1998
Alkaline hydrolysis	YES	Enzymatic	GLUCOSE	GOPXOD	*Synechococcus*	Guerra et al., 2013
Alkaline hydrolysis	YES	Enzymatic	GLUCOSE	GOPXOD	*Synechococcus*	Xu et al., 2013
Alkaline hydrolysis	YES	Enzymatic	GLUCOSE	GOPXOD	*Synechococcus*	Qiao et al., 2018
Alkaline hydrolysis	YES	Enzymatic	GLUCOSE	GOPXOD	*Synechocystis*	Namakoshi et al., 2016
Alkaline hydrolysis	YES	Enzymatic	GLUCOSE	GOPXOD	*Synechocystis*	Mo et al., 2017
Alkaline hydrolysis	YES	Enzymatic	GLUCOSE	HK-G6PD	*Spirulina*	Phélippé et al., 2019
Alkaline hydrolysis	YES	Enzymatic	GLUCOSE	HK-G6PD	*Chroococcidiopsis*	Almon and Böger, 1988
Alkaline hydrolysis	YES	Enzymatic	GLUCOSE	HK-G6PD	*Spirulina*	Aoyama et al., 1997
Alkaline hydrolysis	YES	Enzymatic	GLUCOSE	HK-G6PD	*Synechocystis*	Burrows et al., 2008
Alkaline hydrolysis	YES	Enzymatic	GLUCOSE	HK-G6PD	*Synechocystis*	Gründel et al., 2012
Alkaline hydrolysis	YES	Enzymatic	GLUCOSE	HK-G6PD	*Synechocystis*	Heilmann et al., 2017
Alkaline hydrolysis	YES	Enzymatic	GLUCOSE	HK-G6PD	*Anabaena*	Ernst et al., 1984
Alkaline hydrolysis	YES	Enzymatic	GLUCOSE	HPLC	*Synechocystis*	Hasunuma et al., 2016
Alkaline hydrolysis	YES	Enzymatic	GLUCOSE	HPLC	*Synechocystis*	Hasunuma et al., 2018
Alkaline hydrolysis	YES	Enzymatic	GLUCOSE	*O*-Toluidine	*Synechocystis*	Klotz et al., 2015
Alkaline hydrolysis	YES	Enzymatic	GLUCOSE	Phenol-sulphuric	*Synechocystis*	Miao et al., 2003
Alkaline hydrolysis	YES	Enzymatic	GLUCOSE	Phenol-sulphuric	*Spirulina*	De Philippis et al., 1992
Mechanical cell disruption	–	Enzymatic	GLUCOSE	GOPXOD	*Anacystis*	Lehmann and Wöber, 1976
Mechanical cell disruption	–	Enzymatic	GLUCOSE	GOPXOD	*Anacystis*	Lehmann and Wöber, 1977
Mechanical cell disruption	–	Enzymatic	GLUCOSE	GOPXOD	*Anacystis*	Lehmann and Wöber, 1978
Mechanical cell disruption	–	Enzymatic	GLUCOSE	GOPXOD	*Phormidium*	Bisen et al., 1986
Mechanical cell disruption	–	Enzymatic	GLUCOSE	GOPXOD	*Synechocystis*	Yoo et al., 2015
Mechanical cell disruption	–	Enzymatic	GLUCOSE	GOPXOD	*Synechocystis*	Li et al., 2016
Mechanical cell disruption	YES	Enzymatic	GLUCOSE	GOPXOD	*Anacystis*	Weber and Wöber, 1975
Mechanical cell disruption	YES	Enzymatic	GLUCOSE	GOPXOD	*Synechocystis*	Yoo et al., 2002
Mechanical cell disruption	YES	Enzymatic	GLUCOSE	GOPXOD	*Synechocystis*	Yoo et al., 2007
Mechanical cell disruption	YES	Enzymatic	GLUCOSE	GOPXOD	*Synechocystis*	AU De Porcellinis et al., 2017
Mechanical cell disruption	YES	Enzymatic	GLUCOSE	GOPXOD	*Synechocystis*	De Porcellinis et al., 2018
Thermolysis	–	Enzymatic	GLUCOSE	GOPXOD	*Synechococcus*	Jacobsen and Frigaard, 2014
Thermolysis	–	Enzymatic	GLUCOSE	GOPXOD	*Synechocystis*	Kudoh et al., 2014
Thermolysis	–	Enzymatic	GLUCOSE	HK-G6PD	*Synechococcus*	Suzuki et al., 2007
Thermolysis	–	Enzymatic	GLUCOSE	HK-G6PD	*Synechococcus*	Suzuki et al., 2010
Thermolysis	–	Enzymatic	GLUCOSE	HK-G6PD	*Synechococcus*	Hickman et al., 2013
Thermolysis	–	Enzymatic	GLUCOSE	HK-G6PD	*Synechococcus*	Ohbayashi et al., 2017
Thermolysis	–	Enzymatic	GLUCOSE	HK-G6PD	*Synechococcus*	Sawa et al., 2019

(*continued on next page*)

Table 1 (*continued*)

Extraction[a]	Precipitation[b]	Hydrolysis[c]	Determination[d]	Quantification[e]	Genus	Reference
Thermolysis	–	Enzymatic	GLUCOSE	HK-G6PD	*Synechocystis*	Hanai et al., 2014
Thermolysis	–	Enzymatic	GLUCOSE	HK-G6PD	*Synechocystis*	Iijima et al., 2015
Thermolysis	YES	Enzymatic	GLUCOSE	HK-G6PD	*Synechocystis*	Kawahara et al., 2016

[a] Method for extraction of glycogen from cells. *Acid hydrolysis:* Incubation of cells with 2.5–5.3% (*v*/v) H_2SO_4 at 100 °C for 0.3–6 h.

[b] Recovery of glycogen by precipitation with cold ethanol (30 min on ice or − 20 °C overnight).

[c] Method for hydrolysis of glycogen. *(−)* Hydrolysis not performed; *Enzymatic hydrolysis*: treatment with AMG from *Aspergillus niger* at 25–95 °C for 0.5–12 h; *Acid hydrolysis*: incubation at 100 °C for 30–60 min with 2–5% (*v*/v) H_2SO_4 or HCl.

[d] Estimated compound in the final step of the assay.

[e] Glucose quantification methods. GOD: LabAssay Glucose method (Wako, Osaka, Japan); GOPXOD: glucose oxidase-peroxidase method (Washko and Rice, 1961); HK-G6PD: hexokinase-glucose-6-phosphate dehydrogenase method (Slein, 1965). Carbohydrates quantification methods. Anthrone method (Seifter et al., 1950; Roe and Dailey, 1966); DNS (Dinitrosalicylic Acid Reagent) method (Miller, 1959); Nelson's method (Nelson, 1944); *O*-Toluidine method (Hultman, 1959); Phenol-sulphuric method (DuBois et al., 1956).

[*] Incubation with HCl; *Alkaline hydrolysis*: incubation of cells with 24–32% (*w/v*) KOH at 90–100 °C, for 60–120 min; *Mechanical cell disruption methods*: ultrasounds, bead mills, homogenizers or French Press; *Thermolysis*: boiling (90–100 °C) of samples in water for 10–40 min after extraction with methanol (5 min at 25 °C or 24 h at − 20 °C).

selectively breaks the glycosidic bonds and releases glucose. Acid hydrolysis also efficiently breaks glycosidic bonds, but it is not specific for glycogen, making enzymatic treatment the preferred method among authors. AMG isolated from *A. niger* is the favourite enzyme for glycogen determination protocols (Johnson and Fusaro, 1964; Johnson et al., 1963; Johnson and Fusaro, 1966), as it is highly specific for glycogen and starch (Pazur and Ando, 1961; 1959; Pazur and Kleppe, 1962).

3.1.4. Methods for quantification of glycogen

The methodology available for the determination of carbohydrates in literature is very extensive and is reflected in the diversity of methods for the quantification step reported for cyanobacterial glycogen. The protocol used for quantification can be specific or not. Approximately 24% of the reported methods apply non-specific procedures for the determination of glycogen or glucose (o-toluidine, anthrone, Nelson's, DNS reagent or phenol-sulphuric method), while most reports apply specific methods, such as HPLC or enzymatic assays (glucose oxidase/peroxidase or hexokinase/G6PD).

In conclusion, alkaline hydrolysis followed by precipitation with ethanol is the most widely used method for extraction and purification of glycogen from cyanobacterial cells. This procedure was first developed by (Pflüger, 1902; Pflüger, 1904), further improved for the quantification of glycogen from liver tissue (Seifter et al., 1950; Good et al., 1933; Somogyi, 1957), and then adapted to quantify bacterial glycogen (Stanier et al., 1959). Moreover, the enzymatic hydrolysis of glycogen with AMG and the subsequent enzymatic determination of glucose by the GOPXOD assay is the most specific and preferred protocol for the quantification of glycogen in cyanobacteria.

3.2. Optimization of the methodology for glycogen determination in cyanobacteria

Since the methodology for the extraction of glycogen from cyanobacterial cells is diverse and they differ in their ability to recover soluble glycogen from cells, it is necessary to compare the different available procedures in order to determine their relative efficiencies and select the most suitable one that may be useful to the scientific community. In this regard, the present work shows a comparison of the different procedures used so far for the different steps involved in determination of glycogen in cyanobacteria and offers an improved and more accurate methodology.

Experiments have been performed using the model cyanobacterium *Synechocystis*, since a greater number of reports on glycogen determination are available.

In order to ensure the selectivity of the methodology, we have selected enzymatic assays both for the hydrolysis of glycogen and for the subsequent quantification of glucose. The enzymatic hydrolysis of glycogen with AMG is chosen, since it is highly specific for glycogen and yields higher glucose recoveries than acid hydrolysis for the same time of incubation (Johnson and Fusaro, 1966; Roehrig and Allred, 1974; Nahorski and Rogers, 1972). Acid hydrolysis has been discarded because it is not specific for glycogen (Passonneau et al., 1974). On the other hand, for the determination of glucose, non-specific methods have been omitted from the analysis and the glucose-specific enzymatic GOPX assay has been selected because it is the most widely used in cyanobacteria.

3.2.1. Analysis of the enzymatic colorimetric GOPXOD assay

Nowadays, the GOPXOD enzymatic method has become a widely used procedure for measuring glucose in different biological samples, and is also included as a component of commercial glycogen assay kits. The coupled enzymatic reaction of glucose oxidase and peroxidase was reported as the first specific enzymatic procedure for the estimation of glucose in biological fluids (Keston, 1956; Teller, 1956). In this system, glucose oxidase produces H_2O_2 from the oxidation of glucose and then peroxidase transfers oxygen from H_2O_2 to the chromogenic oxygen acceptor *o*-dianisidine. The oxidised *o*-dianisidine develops a brown colour proportional to the amount of glucose present in the sample (Huggett and Nixon, 1957). Therefore, the absorbance of oxidised *o*-dianisidine at a wavelength of 420 nm shows a linear relationship with glucose concentration. Subsequent studies showed that the colour of *o*-dianisidine is pH-dependent, and that the oxidised product shows three absorption peaks depending on the pH of the sample (McComb and Yushok, 1958). At pH 4.0–7.0, the spectrum exhibits a broad absorption band with a maximum at 450 nm, but when the acidity has a pH 2.0, the absorption peak shifts to 390 nm (McComb and Yushok, 1958; Saifer and Gerstenfeld, 1958). The addition of sulphuric acid at 26% shifts the maximum to 540 nm, changing the colour of the reaction mixture from amber to deep pink. Other authors confirm this observation and demonstrate that the addition of sulphuric acid maintains colour stability for several hours and increases the sensitivity of the colorimetric procedure (Washko and Rice, 1961; Blecher and Glassman, 1962). This principle has been maintained and incorporated into the assay kits for the determination of glucose based on the coupled enzymatic assay of GOPXOD, so the addition of sulphuric acid is widespread in the glucose determination assays. However, it is common to find authors who avoid the sulphuric acid addition, as it is described in the original publications (Keston, 1956; Teller, 1956), and different wavelengths have been used to estimate the oxidised *o*-dianisidine (Johnson and Fusaro, 1966; Roehrig and Allred, 1974), some of them recently reported (Jacobsen and Frigaard, 2014; Kudoh et al., 2014). In the present work, we have

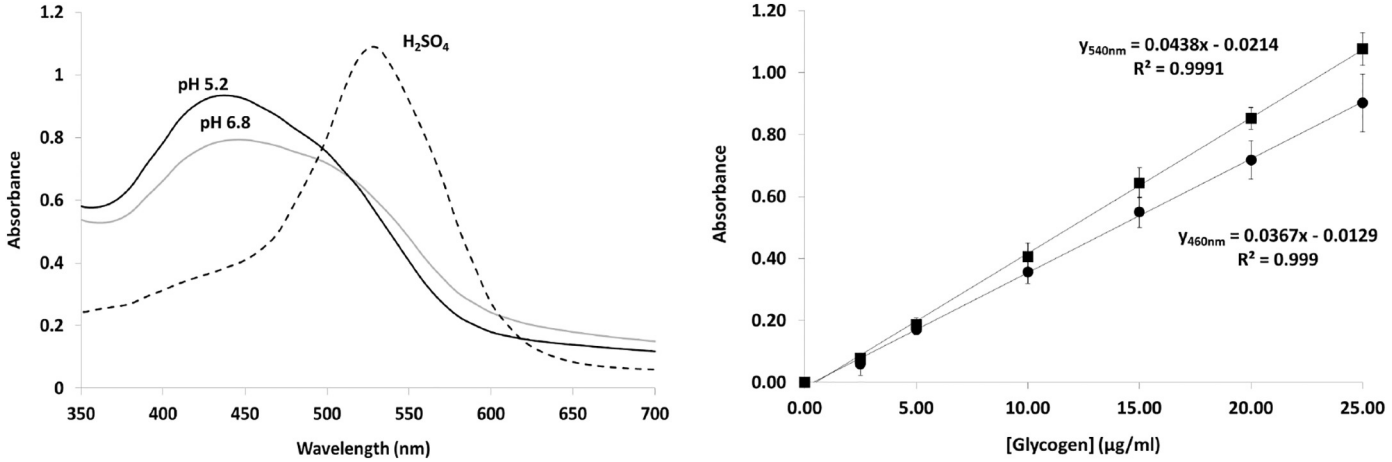

Fig. 2. A) Absorption spectra of GOPXOD-AMG reaction mixtures using standard glycogen. A standard solution of 20 µg glycogen dissolved in sodium acetate pH 5.2 (black solid line) or in TRIS-HCl buffer pH 6.8 (gray solid line) is subjected to the GOPXOD-AMG assay and the absorption spectrum is measured. The same procedure is applied and sulphuric acid is added to final concentration of 4.8 N (dashed lines). B) Linearity of standard glycogen reference curves. Standard glycogen solutions at different concentrations are treated with the GOPXOD-AMG reaction mix at 37 °C for 30 min. Absorbance is measured at 460 nm (circle) or 540 nm (square) in the absence or presence of 4.8 N H_2SO_4, respectively. The mean values of four independent experiments are shown (bar errors represent the standard deviation).

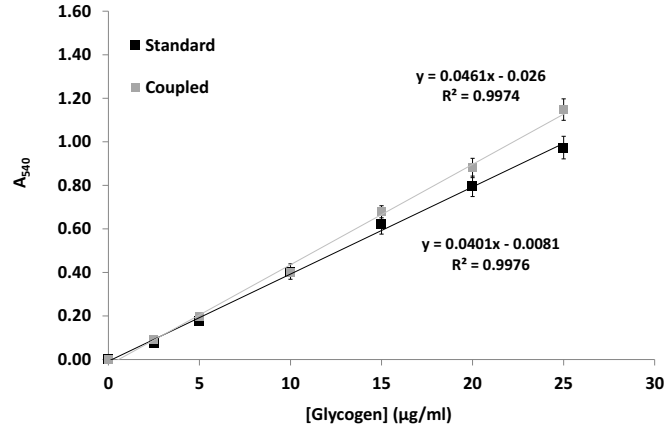

Fig. 3. Glycogen reference curves treated with the "standard" two-step enzymatic assays or the "coupled" one-step enzymatic assay. Standard solutions of glycogen at different concentration are treated with AMG for 2.5 h at 55 °C and followed by treatment with GOPXOD mixture for 30 min at 37 °C (gray) or directly incubate with a reaction mix containing GOPXOD-AMG at 37 °C for 30 min (black). After addition of sulphuric acid, the absorbance is measured at 540 nm. The mean values of three independent experiments are shown (bar errors represent standard deviation).

tested the colorimetric measurement of oxidised *o*-dianisidine at different pH values: 5.2, 6.8 and pH < 2 (Fig. 2). Under conditions for enzymatic assays (pH 5.2 or 6.8), the peak of absorbance of the oxidised *o*-dianisidine is detected at 420–460 nm range and after the addition of sulphuric acid, the peak is shifted to 540 nm. On the other hand, linearity is studied for two wavelengths (460 nm vs. 540 nm) and a linear regression model is applied to standard glycogen calibration curves by measuring absorbance at 460 nm or 540 nm (Fig. 2B). The results are consistent with previous work (Washko and Rice, 1961; McComb and Yushok, 1958), showing that the addition of sulphuric acid slightly increases the sensitivity of the protocol, but the linearity of the assay is similar for both wavelengths (R^2 = 0.999).

The colour of oxidised *o*-dianisidine at 460 nm is stable for at least 2 h (data not shown), similar to reported results obtained with oxidised *o*-dianisidine at 420 nm (Huggett and Nixon, 1957). Researchers can choose between adding sulphuric acid or not to their GOPXOD reactions for glucose quantification. For cultures with low glycogen content, the use of sulphuric acid may improve the sensitivity of the procedure, allowing detection of lower quantities of glycogen.

3.2.2. A straightforward modification to improve enzymatic assay for glycogen determination

We have investigated a coupled enzymatic system which allows the simultaneous enzymatic hydrolysis of glycogen and the enzymatic determination of glucose, in order to reduce the duration of the assay. The AMG, GO and PX enzymes used in the assays for quantification of glycogen operate in the same pH range. AMG from *A. niger* is highly stable in the pH range of 2.3–6.2 (Riaz et al., 2012), with commercial preparations showing a pH optimum from 3.5 to 5.0 (Ono et al., 1988). The pH optimum for GO from *A. niger* and for PX from horseradish are 5.5 (pH range 4–7.2) (Pazur and Kleppe, 1964) and 4.5 (pH range: pH 4–7.5) (Munoz-Munoz et al., 2007), respectively. Reference curves using standard glycogen have been performed comparing the standard procedure of glycogen quantification consisting in treatment for 2 h at 55 °C with AMG followed by treatment with GOPXOD mixture (two-step) for 30 min at 37 °C, with a coupled assay consisting in the simultaneous treatment with a mixture of AMG-GOPXOD (one-step) for 30 min at 37 °C at pH 5.2 (Fig. 3).

The linearity (R^2 value) is similar for both assays in the measurement range and, moreover, the coupled assay yields higher values for glycogen estimation than those of the standard assay (Fig. 3, Supplementary Table 2). Both the systematic errors and the random errors obtained for the coupled assay are below those obtained for the standard assay (Supplementary Table 2). Therefore, the coupled assay is more accurate than the standard assay, since it shows more trueness (closeness to the true value) and precision (repeatability).

To the best of our knowledge, the one-step assay has only been reported for the determination of glycogen in *Anacystis nidulans* by (Lehmann and Wöber, 1976; 1978; 1977). The authors incubated samples for 1 h with a specific glucoamylase/glucose oxidase/peroxidase reagent and then measured the O.D. at 540 nm. There are no other reports describing this coupled assay for cyanobacteria. We propose to perform quantification of glycogen using the one-step coupled assay, as it is more accurate and also significantly diminishes the duration of the procedure.

3.2.3. Procedures for extraction and purification of glycogen in Synechocystis

We have tested and compared the different procedures used for the extraction of glycogen from cyanobacteria to determine their accuracy.

Alkaline hydrolysis, mechanical disruption of cells and thermolysis

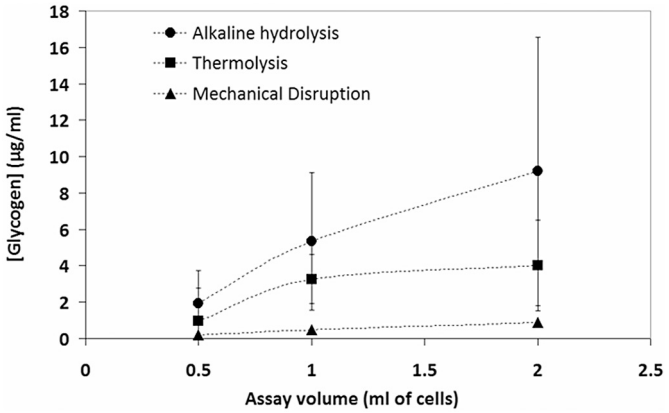

Fig. 4. Comparison of different extraction methods for glycogen quantification. The cultures are recovered at stationary phase (6–8 µg/ml of chlorophyll) and 0.5, 1.0 and 2.0 ml are used for the extraction of glycogen and the subsequent quantification by the "coupled" assay. The mean of three independent extractions from the same biological sample is shown (bar errors represent the standard deviation). The extraction procedures are described in Materials and Methods.

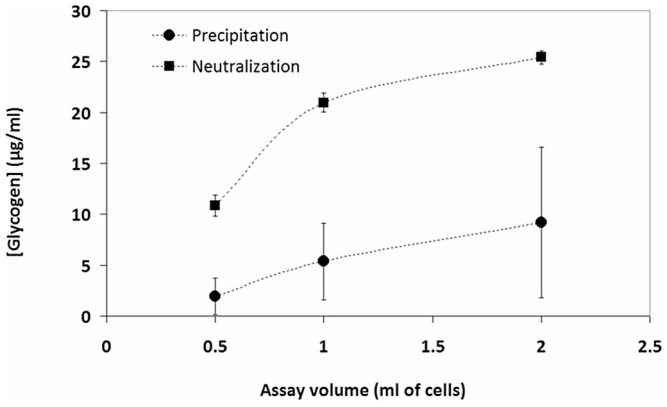

Fig. 5. Comparison of neutralization and precipitation steps after alkaline hydrolysis for glycogen quantification. Cultures in stationary phase (6–8 µg/ml of chlorophyll) are recovered by centrifugation, and 0.5, 1.0 and 2.0 ml are used for the extraction of glycogen and subsequent quantification. The samples are subjected to alkaline hydrolysis and two procedures are compared for the quantification of glycogen: neutralization and precipitation (see M&M). The mean of three replicates of the same biological sample is shown for both procedures (bar errors represent the standard deviation).

procedures have been compared for their ability to extract glycogen from cells, while acid hydrolysis has been omitted from the analysis because, after acid incubation, glycogen is completely hydrolysed towards glucose, preventing the specificity of the procedure(Tebb, 1898).

Cultures at stationary growth phase have been used to test the different extraction methods. Three culture volumes have been used to test for the linearity of the different extraction assays (Fig. 4). Alkaline hydrolysis was followed by the subsequent recovery of glycogen using precipitation in cold ethanol, while for thermolysis and mechanical disruption of cells, glycogen was measured directly in the water-soluble fraction, since they are the most commonly used methods reported in the literature.

Alkaline hydrolysis followed by ethanol precipitation procedure allows the recovery of glycogen to a greater extent than mechanical disruption of cells or thermolysis, resulting in glycogen yields at least one order of magnitude higher. These results suggest that glycogen is present in the water-insoluble fraction when the extraction is performed by physical procedures (mechanical disruption or thermolysis), being alkaline hydrolysis the only extraction method that allows its solubilisation and subsequent recovery by precipitation.

Almost all reports that use mechanical cell disruption comprise the recovery of glycogen from the supernatants after separation of the cellular debris from the soluble fraction. Only one report quantifies the glycogen contained in the insoluble fraction after cell disruption. (Yoo et al., 2002) recovered insoluble glycogen from cell debris by solubilisation with DMSO and subsequent precipitation with ethanol, showing that at least 26% of total glycogen is present in the water-insoluble fraction. They also demonstrated that the solubility of glycogen depends on its internal structure (percentage of branching), which in turn depends on the growth conditions (Yoo et al., 2007). Thus, the procedure involving mechanical cell disruption followed by the recovery and quantification of glycogen from the supernatants allows the quantification of the water-soluble glycogen, underestimating total glycogen content. On the other hand, although thermolysis results in higher values than mechanical cell disruption procedures, probably for its contribution to glycogen solubilisation, the values are significantly lower than those obtained with alkaline hydrolysis.

Our results demonstrate that the methods based on mechanical disruption of cells or thermolysis are too biased, since they quantify only a fraction of the total glycogen and are not recommended.

Alkaline hydrolysis followed by precipitation with cold ethanol is the procedure that estimates the glycogen content of cyanobacterial cells closest to the true value. However, the precision of the method is very low, as the statistical variability of the results for the same experimental conditions is high, indicating a low level of repeatability.

3.2.4. Neutralization with acetic acid vs. precipitation with ethanol after alkaline hydrolysis

A measurement system must be precise to be considered valid. The variability of alkaline hydrolysis plus precipitation with ethanol as a procedure to recover glycogen from cells is high and might depend on experimental errors. It has been reported that small pellets of glycogen can be easily lost during washing and centrifugation along the precipitation step, contributing to increase the variability of results (AU De Porcellinis et al., 2017); personal experience). Moreover, the concentration and the nature of electrolytes present in the sample influence on the solubility of glycogen (Kerly, 1930), and thus, affect the efficiency of the precipitation step.

To avoid possible losses of glycogen during washing steps or in precipitation efficiency, the later has been omitted and samples after alkaline hydrolysis have been subjected to a neutralization step. Samples dissolved in 30% KOH have an extremely high pH value that

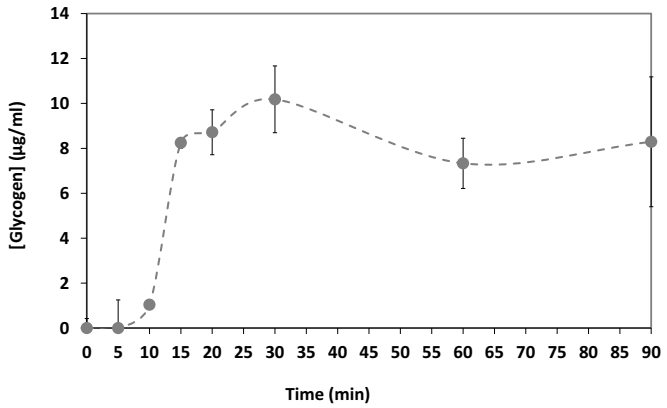

Fig. 6. Effect of the incubation time during alkaline hydrolysis on glycogen quantification. Cultures in stationary phase (6–8 µg/ml of chlorophyll) are recovered and 0.5 ml is used for the extraction of glycogen and the subsequent quantification. Samples are subjected to alkaline hydrolysis at different times and, after neutralization, the glycogen is quantified. Mean of three replicates of the same biological sample are shown (bar errors represent the standard deviation).

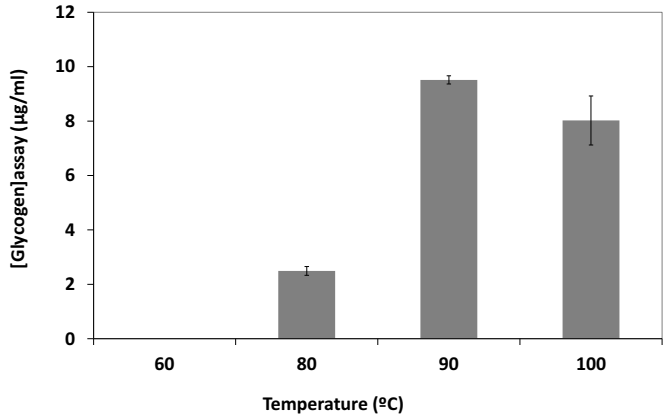

Fig. 7. Effect of hydrolysis temperature on glycogen quantification. Cultures in stationary phase (6–8 µg/ml of chlorophyll) are recovered and 0.5 ml is used for the extraction of glycogen and the subsequent quantification. The samples are subjected to alkaline hydrolysis for 30 min and after neutralization with acetic acid, the glycogen is quantified. Mean of four replicates of the same biological sample are shown (bar errors represent the standard deviation).

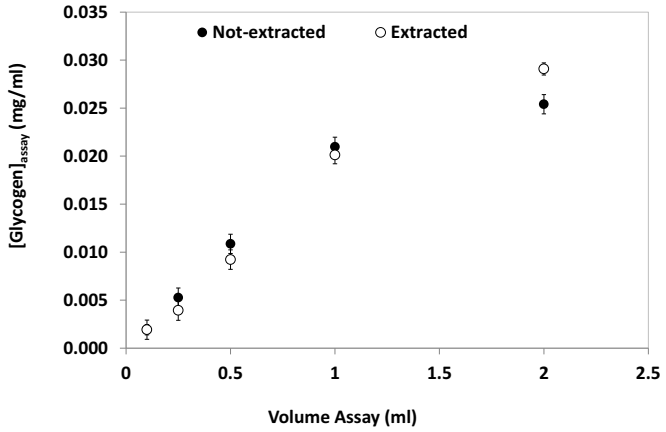

Fig. 8. Effect of pigment extraction on glycogen quantification. Cultures in stationary phase (6–8 µg/ml of chlorophyll *a*) are recovered and 0.5 ml is used for the extraction of glycogen and the subsequent quantification. Samples are subjected to lipid extraction with chloroform: methanol 2:1 (extracted) or use directly for the glycogen extraction (not-extracted). After alkaline hydrolysis for 30 min at 90 °C, samples are cooled at room temperature and neutralized with acetic acid. Afterwards, glycogen is quantified by incubation of neutralized samples with GOPXOD-AMG mix for 30 min at 37 °C. Mean of four replicates of the same biological sample are shown (bar errors represent the standard deviation).

prevents treatment with enzymatic assays. Neutralization by adding acetic acid to the samples can lower pH to a desired value, which allows the enzymatic treatment to be carried out efficiently. In the present work, the volume of acetic acid has been carefully calculated to reach an optimal pH range for the enzymatic assay (Supplementary Table 1). The protocol is described in Materials and Methods.

To test the neutralization performance, an experimental design has been made to compare neutralization and precipitation as alternative steps after alkaline hydrolysis. Samples from the same culture have been subjected to both alternative protocols and a statistical analysis has been performed (Fig. 5).

As shown in Fig. 5, neutralization of the sample after alkaline hydrolysis produces higher recovery values than precipitation of glycogen with cold ethanol. Repeatability is also significantly improved by neutralization. These differences indicate that precipitation with cold ethanol to recover glycogen, not only diminish the precision of the procedure, but also diminish the accuracy, since a fraction of total glycogen is lost during centrifugation and washing steps.

3.2.5. Adjusting incubation time for alkaline hydrolysis

To reduce time consumption of the overall procedure, we have tested different incubation times for alkaline hydrolysis. Fig. 6 shows that complete solubilisation of glycogen is achieved after 30 min and that additional treatment does not produce higher glycogen yields, but an increase in the turbidity of the samples, which interferes in the measurement of absorbance. For this reason, we recommend 30 min as optimal time for alkaline hydrolysis, which significantly reduces the time consumption of the protocol, while increasing the precision (the standard deviation is greatest at 90 min).

3.2.6. Optimum temperature for alkaline hydrolysis

To minimize the evaporation of samples during alkaline hydrolysis, we have tested the effect of temperature on glycogen extraction. We incubate cells for 30 min in the presence of KOH at 60, 80, 90 and 100 °C and, after neutralization of the samples, we have measured the glycogen yields (Fig. 7). Higher yields are reached for samples incubated at 90 °C. This temperature not only shows better performance than 100 °C (more repeatability) but it also avoids the evaporation of samples.

3.2.7. Optimization by previous extraction of non-polar pigments

The optimized procedure for glycogen determination as described above has been tested with different amounts of cells in order to determine the detection limits for cyanobacterial samples. Moreover, as the procedure involves the colorimetric determination of glycogen at the final step, we have also analysed the effect of the extraction of non-polar pigments on measurement, by removing pigments such as chlorophyll *a* and carotenoids, which could interfere with the absorbance (Fig. 8).

Regardless previous extraction is included or not, the optimized procedure is linear in the range from 2.5 to 20 µg of glycogen, showing an upper limit slightly below that of the standard glycogen (Fig. 3). This is probably due to interferences from cell components other than glycogen in the procedure. For the culture used in this experiment (6–8 µg/ml of chlorophyll *a*), the interference of cellular pigments is only significant above the upper limit. From these results, we propose to include a previous extraction step to remove non-polar solvents for cells with low glycogen: chlorophyll ratio.

Table 2
Glycogen accumulation under different nutritional conditions.

Time (h)	Control	P starvation	N starvation	S starvation
	Glycogen (µg/ml)			
0	2.68 ± 0.32	2.68 ± 0.32	2.68 ± 0.32	2.68 ± 0.32
6	10.08 ± 1.14	26.31 ± 2.08	130.86 ± 0.62	122.48 ± 2.91
24	18.23 ± 0.62	37.14 ± 0.01	194.81 ± 2.74	189.18 ± 3.98
48	93.33 ± 7.49	67.53 ± 0.87	200.36 ± 4.86	172.09 ± 4.98
	Chl *a* (µg/ml)			
0	3.00	3.00	3.00	3.00
6	3.65 ± 0.05	2.93 ± 0.12	2.58 ± 0.04	2.79 ± 0.01
24	17.12 ± 0.12	2.99 ± 0.11	2.48 ± 0.04	2.43 ± 0.01
48	36.19 ± 0.04	2.77 ± 0.03	2.38 ± 0.18	2.46 ± 0.01

Cells of *Synechocystis* grown under standard conditions were subjected to different nutrient starvations for 48 h. Glycogen and chlorophyll contents were determined at the indicated times. Glycogen was quantified as indicated in Table 3.

3.2.8. Validation of the protocol under different growth conditions

The protocol has been developed for the cyanobacterium *Synechocystis* grown under standard growth conditions. To further validate the protocol, an analysis of the ability of the proposed methodology to detect significant differences in the glycogen content within the same experiment has been performed. Cells have been grown under

Table 3
Fast protocol for quantification of glycogen in *Synechocystis*.

1	Centrifugate samples at 13 rpm for 1 min
2	Remove supernatant and resuspend cell pellet in 50 µl H_2O
3	Add 200 µl KOH 30% (v/v)
4	Incubate samples at 90 °C for 30 min
5	Add 80 µl glacial acetic acid
6	Allow samples cooling at room temperature
7	Add an enzymatic mix containing AMG, GO, PX and OD in sodium acetate buffer 2.5 mM pH 5.2 to a final volume of 1 ml[a]
8	Incubate samples at 37 °C for 30 min
9	Add H_2SO_4 to a final concentration 4.8 N and measure absorbance at 540 nm (or directly measure absorbance at 460 nm without addition of sulphuric acid)

[a] Final volume can be modified depending on the equipment used for measuring absorbance.

different nutritional conditions that induce glycogen accumulation, such as nitrogen, phosphorous and sulphur deprivation (Table 2) and glycogen has been determined at different times after nutrient starvation. A control experiment by culturing cells under standard conditions (nutrient sufficiency) has also been performed.

As shown in Table 2, glycogen accumulation can be accurately detected by using the improved protocol developed in this work. Differences in the extent of accumulation rates under the different growth conditions tested can also be clearly distinguished, thus providing evidences for the suitability of the proposed protocol to perform quantitative analysis of glycogen in *Synechocystis*.

4. Conclusion

We have comprehensively analysed the literature on the procedures for the determination of glycogen in cyanobacteria concluding that there is an enormous diversity in the methodology applied among researchers. After weak point analysis and performing a series of experiments to optimize the procedure, an improved and novel protocol has been developed that shows the greatest simplicity and accuracy among all those reported so far. The protocol includes a series of steps that are shown in Table 3. The overall processing time per sample is less than two hours, which is a substantial reduction in the duration of reported methods. The protocol could be successfully adapted to other cyanobacterial strains.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.105686.

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Author contributions

R.V.V. and M.V.C. conceived and designed the study, performed the experiments and interpreted results and wrote the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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