Rapid method for the assessment of cell lysis in microalgae cultures

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Abstract A solid understanding of the effect of hydrodynamic forces encountered by microalgae in bioprocesses would benefit existing bioprocesses, eventually allowing an increase in their productivity. For this purpose, a sensitive method able to quantify cell lysis is crucial. Most of the available protocols and methods intended for this purpose were developed for animal or insect cells. In the case of microalgae, the commercial kits tested were unable to determine the cell lysis extension. The method proposed here was developed to relate the release of a cytoplasmic component (enzyme lactate dehydrogenase (LDH)) with cell lysis by measuring the NADH (reduced form of nicotinamide cofactor adenine dinucleotide) produced by LDH. Although different commercial kits based on similar processes are available, they are more complicated to use and not applicable to microalgae nor when longer-term tests are to be performed.

Keywords Cell lysis · Viability · LDH · Microalgae · Dinoflagellate

Introduction

Microalgae are used in different bioprocesses for biofuel production (Chisti 2007), bioremediation (Kraemer et al. 2004; de-Bashan and Bashan 2010; Doria et al. 2012), and CO_2 capture (Aishvarya et al. 2012; González-López et al. 2012) and can provide numerous high-value products (Singh et al.

2005; Camacho et al. 2007; Gallardo-Rodríguez et al. 2012). Pneumatically agitated photobioreactors have proved useful for microalgal culture, but certain algae are damaged by hydrodynamic stress in these systems (Gudin and Chaumont 1991; Jaouen et al. 1999; Chae et al. 2006; Camacho et al. 2011). Shear sensitivity of algal cultures can severely restrict attainable productivity in photobioreactors (Gallardo Rodríguez et al. 2009).

The most common methods for quantifying cell viability are based on the ability of the membrane of viable cells to exclude certain dyes such as trypan blue or propidium iodide (Moore et al. 1998). Viable cells are not stained with such dyes. In contrast, dead or non-viable cells, which have lost the integrity of the cell membrane, allow the passage of these dyes to the cytoplasm where they bind to cell organelles or intracellular molecules. When the cell membrane integrity is compromised, an intracellular material may be released to the medium. For mammalian, insect, or bacterial cells, a wide range of rapid methods have been successfully developed in recent years to facilitate drug discovery screenings (Kepp et al. 2011). However, the majority of these methods cannot be used for microalgae because of the autofluorescence of photosynthetic pigments and their thicker cell coverings. A few methods, based on enzymatic activity of viable cells, partially overcome these hurdles. These methods include those based on fluorescein diacetate (FDA) (Dorsey et al. 1989) and SYTOX Green (Sato et al. 2004), but they are unable to detect cell lysis. If the studied factor or agent is of physical nature (e.g., hydrodynamic stress in bioreactors), non-viable damaged microalgal cells could rapidly disappear before being detected (García Camacho et al. 2007). Alternatively, there are methods based on the fact that non-viable cells, with lost cell integrity, release certain cytoplasmic components. The measuring of the concentration of these components in the broth allows assessing the intensity of the cell lysis produced.

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An important number of these methods are based on the oxidation of lactate to pyruvate by the action of the lactate dehydrogenase (LDH) enzyme and the reduction of the oxidized form of nicotinamide cofactor adenine dinucleotide (NAD$^+$) to its reduced form (NADH). Coupled with this reaction, other compounds may be added. Their change of state can be followed by absorption spectrophotometry or fluorometry. A representative example has been previously described (Bergmeyer and Bernt 1965). The method quantified the LDH released by dead cells by measuring the oxidation of β-NADH to β-NAD$^+$ when LDH reduces pyruvate to lactate. Conversion of β-NADH to β-NAD$^+$ can be monitored by a decrease in absorbance at 340 nm. This decrease is then proportional to the amount of LDH present. Based on similar techniques, it can be found that there are a number of commercial kits for determining breakage or cell lysis. Most of these kits require a two-step procedure to remove a culture medium and, therefore, are not homogeneous. Non-homogeneous processes are not recommended for intensive screening programs due to the large amount of sample handling they usually need.

The above-described protocols were developed for animal and/or insect cells and are not directly applicable to microalgae. The presence of pigments and a lower concentration of intracellular LDH in microalgae results in the inapplicability of these methods, compared to animal cells.

In this work, a cost-effective, rapid, and sensitive method was therefore proposed for assessing viability or cell death through indirect determination of the enzyme LDH released into broth after the loss of the integrity of the cell membrane. The methodology is not only adequate for microalgae but also for any other types of cell line. The reagents required were proven innocuous to cells allowing long-term in situ monitoring of cell lysis.

Materials and methods

Cells and growth conditions

Monocultures of the microalga *Tetraselmis suecica* and the dinoflagellate microalga *Protoceratium reticulatum* were used. *T. suecica*, strain CCAP 66/4 (obtained from Culture Collection of Algae and Protozoa (CCAP)), was cultured in 0.5-L flasks at 23 °C with enriched seawater (ES) (Berges et al. 2001) under continuous illumination (75 μmol photons·m^{-2}·s^{-1}) and aeration at 0.2 vvm.

Two strains of *P. reticulatum* were used. Strain GG1AM was obtained from the Culture Collection of the Centro Oceanográfico, Vigo, Spain. The other strain, CCMP3241, was obtained from the Culture Collection of the Bigelow Laboratory for Ocean Sciences, Maine, USA. Both were grown in Erlenmeyer flasks in an L1 medium (Guillard and Hargraves 1993) under a 12:12-h light-dark cycle (100 μmol photons·m^{-2}·s^{-1}) at 18 °C.

To reinforce the reliability of the measurement method proposed for microalgae, its use with different types of animal cells was further tested: mouse T lymphocyte cells (line EL-4) and SE-UCR insect cells. EL-4 cells were grown in Roswell Park Memorial Institute medium (RPMI 1640)-10 % fetal bovine serum (FBS) (Sigma-Aldrich; R8758). Cells were maintained in 25 cm^2 T flasks (Sigma-Aldrich; Ref. F7177) at 37 °C in an incubator. The insect cells were grown in TNM-FH-10 % FBS (Sigma-Aldrich; T3285). Cells were maintained in 25 cm^2 T flasks (Sigma-Aldrich; F7177) at 27 °C in an incubator.

Rationale of the method

The developed protocol (Spanish National Patent no. 201300775) was used to determine the cytoplasmic enzyme LDH in the supernatant of cultures where a part of the cell population was dead and/or lysed. The method allows the indirect measurement of LDH concentrations ranging from 0.0001 to 0.001 U mL^{-1}. The reagent mixture consists of lactate and the cofactor NAD$^+$ and may contain a carbonate-bicarbonate buffer.

When lactate is used as a substrate and the enzyme LDH is the cellular component to be measured, the enzyme initiates a reaction in which lactate (included in the reagent mixture) is oxidized to pyruvate. This reaction also requires NAD$^+$. The oxidation of lactate to pyruvate results in the production of NADH. The final result is then a method in which a cytoplasmic component, the LDH enzyme released by dead or non-viable cells, is quantified indirectly by the generation of NADH that is determined fluorometrically. As will be shown later, the production of NADH is proportional to the amount of enzyme LDH present. Therefore, a calibration curve might be obtained which relates the amount of NADH produced with the amount of LDH enzyme present in a given sample volume curve. The final result is then a method in which a cytoplasmic component, the LDH enzyme released by dead or non-viable cells, is quantified indirectly by the generation of NADH that is determined fluorometrically.

Cell counts

Animal cells were counted in a hemocytometer (Neubauer chamber) under a light microscope, and cell viability was measured using the trypan blue dye exclusion method. Microalgae were fixed with Lugol's solution, and the cells were counted on a Sedgewick-Rafter counting slide.

Determination of LDH in lysed cell samples

Serial dilutions of viable cell samples (>97 % of viability) were prepared using the appropriate culture media as diluents. Cells were completely lysed, applying ultrasound (cycle of 0.5 and amplitude of 80 % in a probe system of 200 W) for 1 min. The extent of cell lysis was determined by light microscopy.

After the lysis step, the samples were centrifuged to remove cell debris. The released LDH (hence, cell lysis) was quantified using the following method:

a. Supernatants (100 μL) were placed in the wells (transparent flat-bottom 96-well black plates; Corning; Sigma-Aldrich).

b. Then, lactate and NAD^+ were added to the sample (90 μL). Both reagents had a concentration of 1.5 mM and were diluted in a carbonate-bicarbonate buffer (final pH ≈ 8.5). Control samples where NAD^+ was not added were carried out to determine (and subtract) any fluorescence source not related with the LDH reaction.

c. Microwell plates were incubated for 20 min at 17 °C (microalgae), at 37 °C for hybridoma cells, and at 27 °C for SE-UCR cells.

d. A 25 % sodium dodecyl sulphate (SDS) solution (60 μL) was added to stop the reaction. The final volume was 250 μL. The final SDS percentage in the reaction volume was 6 %.

e. The protocol implies the measurement of the emitted fluorescence of the NADH formed. It was read using a monochromator-based microplate reader (Synergy Mx, BioTek). The excitation and emission wavelengths were 353 and 450 nm, respectively.

Steps from a to e were also applied to non-lysed viable cells, gently centrifuged to avoid any damage. It was observed that, when viability was still near 100 %, no LDH was released even at relatively high cell concentrations (>10^6 cells mL^{-1}).

Two types of calibration curves were performed. The first one was based on the number of broken/lysed cells, and the second one was based on LDH units for which LDH (lyophilized powder, 140 U mg^{-1}) was purchased from Sigma-Aldrich (L1254). The rest of reagents required for the method were lactate (L7022; Sigma-Aldrich), NAD^+ (N7004; Sigma-Aldrich), NADH (N4505; Sigma-Aldrich), and sodium dodecyl sulfate (436143; Sigma-Aldrich).

Statistical analysis

Experimental error was determined from triplicate assays, and standard deviation was calculated. Comparison of treatments over time was performed using the nonparametric paired sign test ($\alpha = 0.05$). Tests were conducted in Statgraphics Centurion XVI (StatPoint, USA).

Results and discussion

Correlation between concentration and fluorescence measurement of NADH It is widely known that NADH is excited at 340 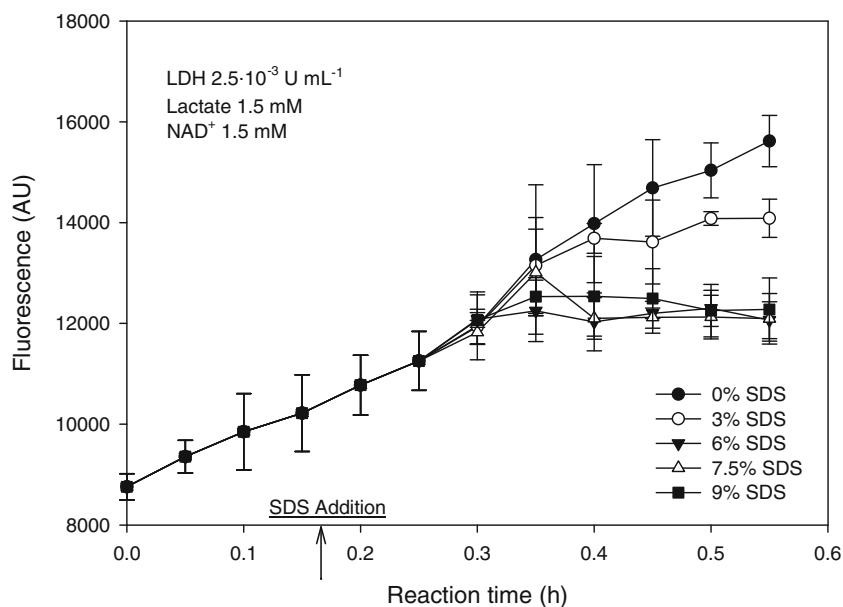or 365 nm and emits fluorescence in the range of 400 to 550 nm with a maximum at 450 nm (Jameson et al. 1989). In contrast, the NAD^+ under these conditions does not fluoresce. To use this property to measure the extent of the reaction catalyzed by LDH, it is necessary to establish the linearity between fluorescence at 450 nm and the concentration of NADH in different solvents or culture media. Three different media were tested showing linearity up to a concentration of 8 nmoles of NADH (data not shown): L1 medium, RPMI-10 % FBS, and TNM-FH-10 % FBS.

Stopping the reaction The possibility of stopping the reaction catalyzed by LDH is an interesting aspect, both for storage of samples and the measurement of a large number of these in a short-time interval. The detention of the reaction was found to be effective by adding an aqueous solution of SDS; 90 μL of the reagent solution was added per well containing the following concentrations: [LDH] = 2.5 × 10^{-3} U mL^{-1}, [lactate] = 1.5 mM, and [NAD^+] = 1.5 mM. After incubating for 10 min at room temperature, 60 μL of the stop solution prepared at different concentrations of SDS was added. In Fig. 1, it can be observed that percentages of SDS in the sample equal to or greater than 6 % were able to stop the reaction 10 min after its addition.

Tests performed with *P. reticulatum* cell lysates (as a source of LDH) were also carried out. Similarly, a volume of 60 μL of 25 % SDS solution was sufficient to stop the reaction in wells containing up to 40,000 cells of *P. reticulatum*-lysed cells in a volume of 90 μL (reagents were provided at the same concentrations).

Effect of operational factors A study of the effect of LDH, lactate, and NAD^+ concentrations on fluorescence emission from the reaction mixture allowed selecting an operating range for each of these factors. In this regard, the lactate concentration tested ranged from 0.075 to 80 mM and NAD^+ from 0.075 to 3 mM. These tests were performed following the complete protocol which included the addition of SDS (stopping reagent). The combination of both concentrations and reaction time made possible the quantification of a wide range of LDH concentrations. Figures 2 and 3 illustrate some of the assays carried out. Since fluorescence continues growing for hours if SDS is not added (see Fig. 4), the reaction time was decided, taking into account both the sensitivity and method's convenience/usability. The reaction time for the method proposed was fixed at 20 min. As shown in Figs. 2 and 3, both lactate and NAD^+ concentrations influenced the production of NADH by the enzyme LDH. In the range assayed, these substrates affected the production of NADH in a proportional fashion. As can be observed in Fig. 3, the method allows the indirect measurement of LDH concentrations ranging from 0.0001 to 0.001 U mL^{-1}. In this figure, the medium basal fluorescence was subtracted from the measured values to establish a calibration curve for the LDH enzyme.

Fig. 1 Effect of sodium dodecyl sulfate (*SDS*) on the kinetics of the reaction catalyzed by LDH in which lactate is oxidized to pyruvate, followed through the reaction product (NADH), whose fluorescence was measured at 450 nm. SDS percentages are final concentrations in the reaction volume

LDH $2.5 \cdot 10^{-3}$ U mL^{-1}
Lactate 1.5 mM
NAD$^+$ 1.5 mM

SDS Addition

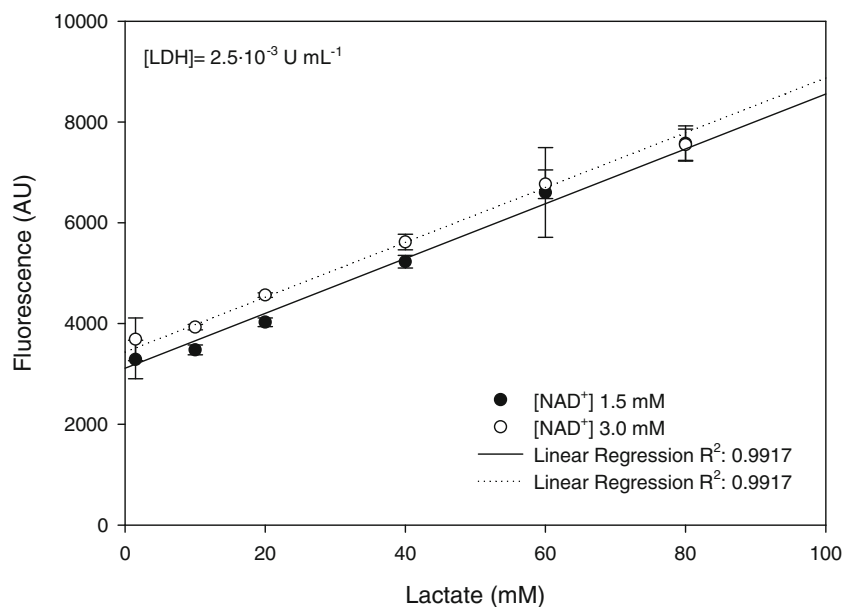- ● 0% SDS
- ○ 3% SDS
- ▼ 6% SDS
- △ 7.5% SDS
- ■ 9% SDS

Determination of optimal pH for LDH activity Any enzyme has an optimal range of action, and outside of which, it shows no or low activity. Therefore, it is necessary to know if the pH of different culture media is able to negatively influence the process involved in this method. For this, the activity of LDH was determined in the pH range of 6.5 to 8.5. This range encompasses the most usual conditions of cultivation of many cell lines. To set pH below 7.5, a phosphate-bisphosphate buffer was used. Higher pH was obtained using a carbonate-bicarbonate buffer (1–100 mM).

Up to a pH value of 8, LDH presented a basal steady activity, but at a pH value of 8.25, it abruptly increased to attain a maximum value that was around four times the baseline value.

Consequently, the buffer proposed in the method was the carbonate-bicarbonate buffer. Although the measurements could be performed at lower or higher pH values, it is advisable to use any buffered medium whose pH is not less than 6.5 or greater than 8.5.

Effect of reaction time on fluorescence of NADH formed Figure 4 shows that, for the L1 medium, if SDS solution is not added, NADH is continuously produced up to 12 h (*p* value <0.05). From 12 h onwards, a fluorescence emission is stable for at least 12 h more (*p* value <0.05) but with increasing dispersion on the data. After freezing the reaction with SDS (6 %) at 30 min, the NADH produced was stable for 24 h. Similarly, when other culture media (RPMI

Fig. 2 Influence of the concentration of NAD$^+$ and lactate in the production of NADH catalyzed by LDH. NADH was determined by measuring the fluorescence emission at 450 nm. SDS was added after 20 min of reaction

[LDH]= $2.5 \cdot 10^{-3}$ U mL^{-1}

- ● [NAD$^+$] 1.5 mM
- ○ [NAD$^+$] 3.0 mM
- — Linear Regression R^2: 0.9917
- ⋯ Linear Regression R^2: 0.9917

Fig. 3 Effect of the concentration of LDH in the production of NADH. NADH was determined by measuring the fluorescence emission at 450 nm. The reaction performed with two different combinations of lactate and NAD$^+$. SDS was added after 20 min of reaction. Basal fluorescence was subtracted from the measured values
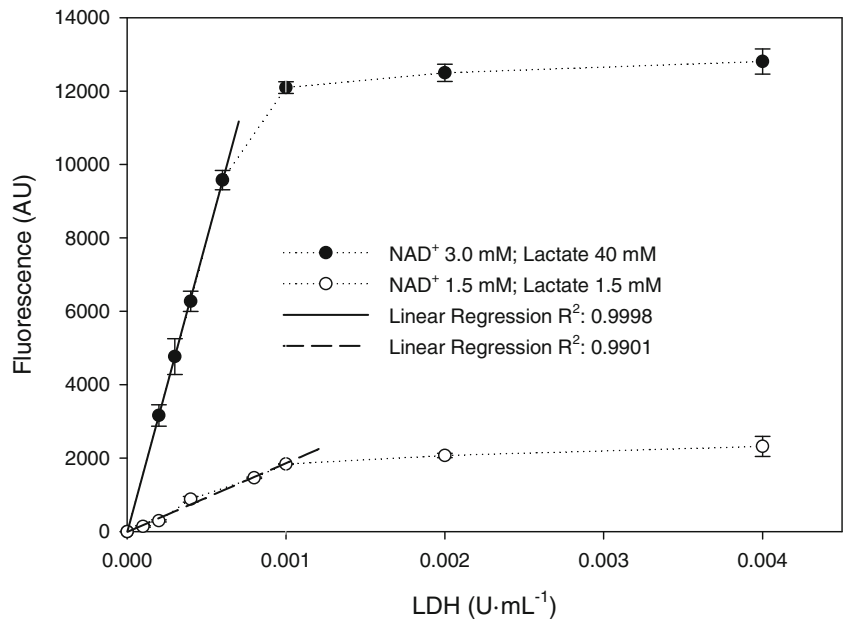
and TNM-FH) were assayed, a stable fluorescence emission was observed. These assays demonstrated that the proposed method can be used to measure cell lysis in situ for extended periods of incubation and that the NADH formed is stable.

Background fluorescence and linearity The following types of cells were used for the tests: (a) the microalga *P. reticulatum*, strains GG1AM and CCMP3241 grown in an L1 culture medium; (b) the microalga *T. suecica* grown in enriched natural seawater; (c) cells of mouse T lymphocytes, EL-4 line, grown in RPMI medium with 10 % FBS; and (c) insect cells, line SE-UCR, grown in TNM-FH with 10 % FBS.

Media assayed shown different levels of basal fluorescence not related to LDH reaction. In addition, the media containing FBS are expected to contain LDH. Serial dilutions of each of the abovementioned cell cultures were prepared using the culture media as the diluent itself. The dilutions were prepared in well plates, making up to a volume of 100 μL. The concentration ranged from 0 to 80,000 cells per well. After this period, the cells were lysed using ultrasounds (see "Materials and methods"). Then, the wells were centrifuged to precipitate cellular fragments. The reagent solution was added as described previously.

Control samples were also included in these assays. They consisted in adding to the lysed cell supernatants all reagents except NAD$^+$. The fluorescence measured in the control

Fig. 4 Production of NADH by LDH in an L1 culture medium with and without a stopping agent (SDS 6 %). NADH was determined by measuring the fluorescence emission at 450 nm
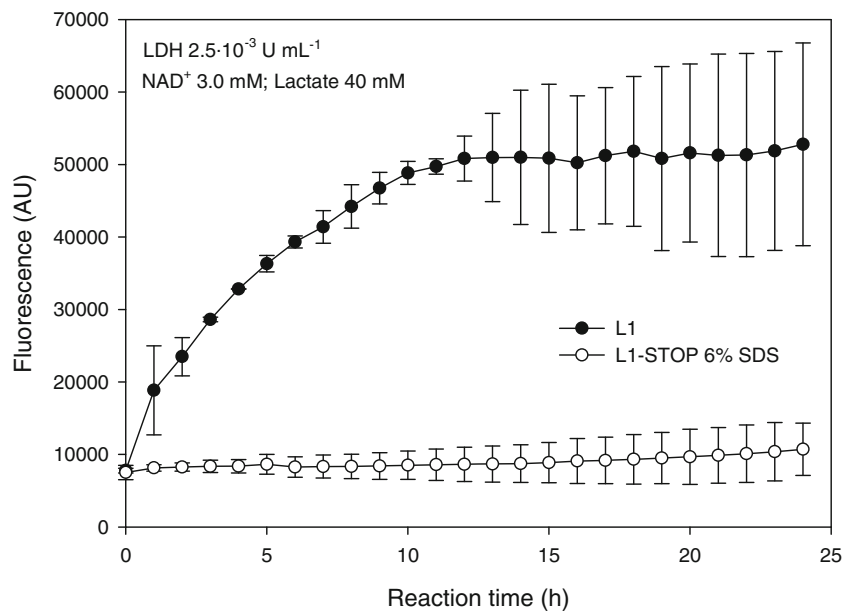
samples was then subtracted to the samples where all reagents were added. Consequently, medium/supernatant florescence and previously released NADH did not influence the LDH determinations. All the wells were incubated for 20 min at the temperatures mentioned in the "Materials and methods" section. Finally, the fluorescence emission from each well was measured (λ_{ex}=353 nm; λ_{em}=450 nm). Figure 5 gathers the results obtained. In all the cases, there was a linear relationship between the fluorescence (hence, concentration of NADH produced by the released LDH) and the number of broken cells. A basal level of fluorescence (tested in the absence of broken cells) that was specific for the culture medium was also observed. The highest value corresponded to the assays carried out on a RPMI medium. Since there is a linear relationship between the percentage of broken cells and the fluorescence, it is advisable to perform a calibration curve, with at least three points, to consider the possible influence of the culture conditions or cell age on the intracellular LDH contents. The protocol was proven adequate for the different cell lines assayed unlike other commercially available colorimetric assays that resulted to their inability to determine LDH in microalgae supernatants. The method is also very simple as it measures the NADH

directly produced by the intracellular LDH without the addition of other intermediate compounds like the tetrazolium salts.

Stability of LDH in culture media LDH enzyme was added to different culture media (without cells or cell lysate) at a concentration of 2.5×10^{-3} U mL^{-1}. The solutions were kept at 37 °C in the case of RPMI+10 % FBS and 17 °C for the L1 medium. Samples were taken at 0, 1, 2, 3, 4.5, and 24 h, and then, the reagent mixture was added ([lactate]=1.5 mM; [NAD$^+$]= 1.5 mM). After the reaction time, SDS (6 % final concentration) was added and fluorescence was measured at 450 nm. LDH activity depended on the medium where the enzyme was dissolved, but it showed a similar behavior. The enzyme activity showed a reduction of 50 % (in 4.5 h) in the case of L1 medium and a 25 % (in 2 h) in the case of RPMI (data not shown). After this reduction, the activity was steady up to 24 h. Since the LDH activity, half-life is high enough and time courses of extracellular lactate dehydrogenase accumulation during batch cultures under cytotoxic or damage conditions would be representative of a cell lysis phenomenon. A similar conclusion was reached in the study of Wagner et al. (1992) where the released LDH was used to estimate cell death and growth in mammal perfusion cultures.
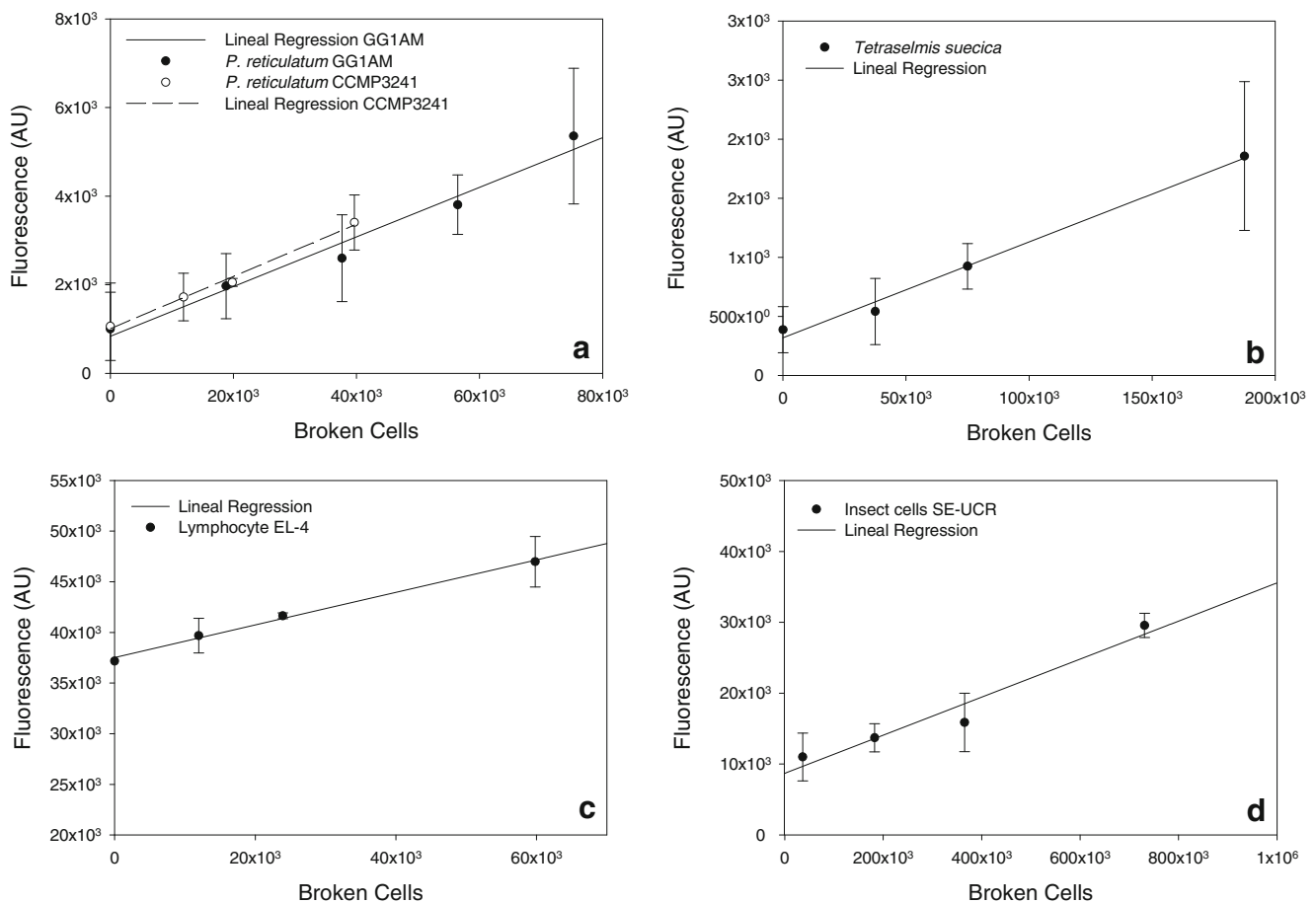
Fig. 5 The relationship between the number of broken cells and the fluorescence emission of NADH produced by LDH for cells of dinoflagellate microalga *P. reticulatum* (CCMP3241 and GG1AM) (**a**), microalga *T. suecica* (CCAP 66/4) (**b**), insect cells (SE-UCR) (**c**), and mouse T lymphocyte cells (EL-4) (**d**). The complete protocol as described in the "Materials and methods" section was followed

Summary of method proposed and applicability The concentration selected for the method was 1.5 mM for lactate and NAD$^+$. A buffer was also advisable to adjust the pH to 8.5 in the reaction volume (a carbonate-bicarbonate buffer is proposed here). Production of NADH was measured after 20 min. The reaction can be stopped using SDS at a final concentration of 6 % (w/v). When used to determine cell death, the method requires incubating cells in a culture medium with a toxic mixture to be tested or for application of any physical disturbance. These agents will cause total or partial lysis on the cell population and, consequently, the release of LDH into the medium. The mixture of reagents used in the method proposed is non-toxic to living cells, so a small volume can be added in situ to cells without affecting the results of the study. In tests where greater volume was the object of study, it is recommended to take into account the addition of the reagents to the sample rather than to the whole culture volume.

The lack of a solid understanding of the effect of hydrodynamic forces encountered by microalgae in bioprocesses has potentially limited the productivity in these systems (Chae et al. 2006; Camacho et al. 2011). In a highly turbulent bioreactor (i.e., bubble column), non-viable microalgae cells would rapidly disappear and the operator could not differentiate growth inhibition (caused by low-light inhibition, waste medium, temperature inhibition, etc.) from cell breakage caused by hydrodynamic forces. The quantification of cell lysis using the method proposed here could assist in the clarification of some of this confusion. Furthermore, process equipment designers (filtration, pumping, etc.) would also benefit from this protocol. As was previously demonstrated, the overall productivity could be greatly affected by the cell damage produced in these devices (Mollet et al. 2004).

Conclusions

The developed protocol is very simple and easy to implement. It was shown successfully in the determination of the cytoplasmic enzyme LDH in the supernatant of cell cultures. It allowed the quantification of dead and/or lysed cells in animal, insect, and microalgae cells. It is proposed as a tool to be used in microalgae bioprocess development and research. The quantification of cell lysis by the method proposed here might assist in achieving a better understanding of the effect of hydrodynamic forces encountered by microalgae in bioprocesses.

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