Bioresource Technology 101 (2010) 7587–7591

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Protein measurements of microalgal and cyanobacterial biomass

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ARTICLE INFO

Article history:
Received 7 January 2010
Received in revised form 12 April 2010
Accepted 22 April 2010
Available online 21 May 2010

Keywords:
Cyanobacteria
Kjeldahl method
Lowry method
Microalgae
Protein content

ABSTRACT

The protein content of dry biomass of the microalgae *Porphyridium cruentum*, *Scenedesmus almeriensis*, and *Muriellopsis* sp. and of the cyanobacteria *Synechocystis aquatilis* and *Arthrospira platensis* was measured by the Lowry method following disruption of the cells by milling with inert ceramic particles. The measurements were compared with the Kjeldahl method and by elemental analysis. The nitrogen-to-protein conversion factors for biomass obtained from exponentially growing cells with a steady state doubling time of ∼23 h were 5.95 for nitrogen measured by Kjeldahl and 4.44 for total nitrogen measured by elemental analysis. The protein content in dry biomass ranged from 30% to 55%. The above conversion factors are useful for estimating the protein content of microalgal biomass produced in rapid steady state growth as encountered in many commercial production processes.

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1. Introduction

Proteins make up a large fraction of the biomass of actively growing microalgae and cyanobacteria, although they are generally undervalued compared to minor products such as omega fatty acids. Protein content of microalgal biomass is determined either by colorimetric methods (Bradford, 1976; Lowry et al., 1951; Smith et al., 1985) or by methods that measure the concentration of elemental nitrogen. Colorimetric assays rely on the appearance of a chromophore as a consequence of either the binding of a dye to protein or the protein being involved in a redox reaction. Colorimetric protein assays have been reviewed by Sapan et al. (1999). Colorimetric assays are sensitive to interferences (Peterson, 1979) and their accuracy depends greatly on the methods used for pretreating the sample (Guerlava et al., 1998). Measurements of nitrogen by Kjeldahl or total nitrogen by elemental analysis are less susceptible to interferences, but nitrogen-to-protein conversion factors must be established to relate the measured nitrogen to the amount of protein.

If all the nitrogen in a biomass sample is associated exclusively with protein, then nitrogen mass fraction measured by the Kjeldahl method can be unambiguously converted to the protein content by multiplying by a nitrogen-to-protein conversion factor value of 6.25. Similarly, the nitrogen content of a sample measured by elemental analysis accurately reflects the protein concentration if the multiplying factor is 6.25 and all the nitrogen is associated with the protein. Biomass usually contains a proportion of nitrogen that is not associated with proteins, but with compounds such as DNA. Algal pigments such as chlorophyll also contain a significant amount of nitrogen and the biomass often contains inorganic nitrogen (Fujihara et al., 2001; Lourenço et al., 1998; Wallace and Fox, 1998). Consequently, the commonly used multiplier of 6.25 causes the protein content to be overestimated (Ezeagu et al., 2002) and biomass specific multipliers have to be determined. For example, conversion factor values of 5.51, 3.59, 5.64 and 3.24 have been proposed for apple flower buds (Khanizadeh et al., 1992), sweet potato (Yeoh and Truong, 1996a), wild fruits from south-eastern United States (Levey et al., 2000) and cassava roots (Yeoh and Truong, 1996b), respectively. Conversion factors for the various macroalgae and microalgae have been claimed to vary from 3.75 to 5.72 (Lourenço et al., 2002, 2004).

For microbial proteins to be measured accurately, the cells must be pretreated to fully release the intracellular proteins. Pretreatments typically involve disrupting the cells by physical or chemical means (Barbarino and Lourenço, 2005; Guerlava et al., 1998). Hydrolytic enzymes and chemicals such as sodium dodecyl sulfate (SDS) can be used to lyse the cell walls (Meijer and Wijffels, 1998), but not all cells are equally susceptible to enzymes and chemicals (Chisti and Moo-Young, 1986). Milling a cell slurry in presence of glass beads or other fine ceramic particles is known to be one of the most effective methods of releasing intracellular proteins from even the hardiest of microbial cells (Chisti and Moo-Young, 1986;

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doi:10.1016/j.biortech.2010.04.077

Garrido et al., 1994). Bead milling is especially effective for microalgae (Chisti and Moo-Young, 1986). In the laboratory, milling generally involves the use of pestle and mortar. Ultrasonication is another effective method of disrupting cells, but has the potential to damage the proteins (Chisti and Moo-Young, 1986) and is therefore unacceptable if bioactive proteins are wanted.

Here we use the well-known Lowry method (Lowry et al., 1951; Peterson, 1979) to directly measure the protein content in the biomass of five different microalgae and cyanobacteria that had been pretreated to fully disrupt the cells. The Kjeldahl method and elemental analysis were then used to measure the Kjeldahl nitrogen and total nitrogen content of the various biomass samples. The directly measured Lowry protein content was then correlated with the measured Kjeldahl and elemental nitrogen content to determine the nitrogen-to-protein conversion factors. Previously reported conversion factors (Lourenço et al., 2002, 2004) had been determined using biomass samples from batch cultures, and the data were reported without regard to the specific growth stage or the physiological state of the culture. In the current study, biomass samples that came from steady state cultures in rapid growth were analyzed. This growth state is more reflective of the state when the microalgae are harvested commercially.

2. Methods

2.1. Biomass and chemicals

The red microalga *Porphyridium cruentum* (UTEX 161), the green microalgae *Scenedesmus almeriensis* (CCAP 276/24) and *Muriellopsis* sp. (Del Campo et al., 2000); and the cyanobacteria *Synechocystis aquatilis* (SAG 90.79) and *Arthrospira platensis* (SAG 85.79) were grown in phototrophic continuous cultures in artificially illuminated (1200 $\mu E\ m^{-2}\ s^{-1}$ irradiance with 12 h light/dark cycle) 2-L bubble columns (0.07 m in diameter, 0.47 m working depth) aerated at a constant rate of 0.5 L min^{-1}. *P. cruentum* was grown in a previously specified (García-Malea et al., 2005) acetate-free inorganic medium. The other microalgae were grown in Mann and Myers culture medium (Mann and Myers, 1968). The cyanobacteria were grown in Zarrouk's modified culture medium (Starr and Zeikus, 1993). All media were nitrogen sufficient. The nitrogen (as nitrate, NO_3^-) concentration in the feed medium was 10.3 and 11.8 mM for *P. cruentum* and the other microalgae, respectively. For cyanobacteria, the feed concentration of nitrogen was 29.4 mM.

The medium in a bubble column was inoculated with the algal culture (~250 mL) that had been grown in round-bottomed flasks (25–27 °C, 100 $\mu E\ m^{-2}\ s^{-1}$ continuous light) using the above-mentioned media. The pH was controlled automatically at 8.0 for the microalgae and 9.5 for the cyanobacteria by sparging with carbon dioxide as needed. Growth was therefore not limited by carbon supply. The temperature was controlled at 25–27 °C. The biomass was allowed to grow in batch mode for 7–10 days, depending on the algal species. The culture was then switched to continuous feeding with the relevant medium at a dilution rate of 0.03 h^{-1} unless specified otherwise. Feeding occurred only during the photoperiod. The culture volume in the bubble column remained constant because of overflow harvest of the broth at the same rate at which the column was fed. A steady state was attained within 7–10 days of switching to continuous operation and maintained for 3 days as demonstrated by a constant biomass concentration in the overflow from the bubble column. The steady state biomass concentration, measured gravimetrically, was around 1 g L^{-1}.

During each steady state, the overflow from the bubble column was collected in a sample tube. More than 500 mL of broth could be collected daily using this procedure. The biomass was recovered by centrifugation (2200*g*, 5 min), washed with a 1% (w/v, g/100 mL) aqueous NaCl solution, centrifuged again and freeze-dried. The dry biomass was analyzed immediately or stored at −22 °C for up to 10-days prior to analysis.

The chemicals used were purchased from Sigma–Aldrich (Sigma–Aldrich Co., St. Louis, MO, USA) and Panreac Química SAU (Barcelona, Spain). The following solutions were prepared in distilled water: (1) lysis buffer (5 mL L^{-1} of Triton X-100, 0.3722 g L^{-1} of ethylenediaminetetraacetic acid disodium salt, 0.0348 g L^{-1} of phenyl methyl sulfonyl fluoride); (2) SDS solution (0.05 g L^{-1} of sodium dodecyl sulfate salt), (3) bovine serum albumin (BSA) solution (0.1 g of dry BSA dissolved in lysis buffer and made up to 50 mL in the same buffer); (4) reagent A (4.0 g L^{-1} of sodium hydroxide and 20.0 g L^{-1} of sodium carbonate); (5) reagent B1 (0.001 g L^{-1} of copper II sulfate pentahydrate); (6) reagent B2 (0.002 g L^{-1} of potassium sodium tartrate tetrahydrate); (7) reagent C (20 mL of reagent A, 0.2 mL of reagent B1 and 0.2 mL of reagent B2 prepared just prior to use); (8) Folin–Ciocalteu reagent (1:1 v/v Folin reagent/distilled water) prepared just prior to use.

2.2. Biomass pretreatment

The following pretreatment methods were tested: (i) suspension in lysis buffer; (ii) ultrasonication at high power for 10-min using a commercial sonic bath (Ultrasons, J.P. Selecta, Barcelona, Spain) in lysis buffer; (iii) milling for 5-min with a pestle and mortar without grinding elements prior to suspension in lysis buffer; and (iv) milling for 5-min with a pestle and mortar in presence of an inert ceramic (aluminum oxide Type A-5, Sigma A2039; Sigma–Aldrich Co., St. Louis, MO, USA) powder as the grinding particles prior to suspending in the lysis buffer. The weight of the grinding particles was the same as the weight of the algal biomass powder.

2.3. Kjeldahl method

Kjeldahl nitrogen (Owusu-Apenten, 2002) was measured (J.P. Selecta 4000508 block digester, J.P. Selecta, Barcelona, Spain; Buchi Kjeldahl K-314 distillation unit, BÜCHI Labortechnik AG, Flawil, Switzerland) using 200 mg samples of the dry biomass.

2.4. Elemental analysis

The total nitrogen content of the freeze-dried biomass was measured by elemental analysis (LECO CHNS-932 with oxygen furnace VTF900, LECO Corporation, St. Joseph, MI, USA). Samples (2 mg) were placed in tin capsules in the oven for combustion at 950 °C using pure oxygen (20 cm^3) as the combustion gas and pure helium as the carrier gas. Carbon, hydrogen and sulfur were determined by infrared absorption while nitrogen was measured as N_2 using a thermal conductivity detection system.

2.5. Lowry method

The Lowry method (Lowry et al., 1951) was used to measure the protein content of the pretreated biomass. For the control pretreatment, 20 mg aliquots of the freeze-dried biomass were suspended for 20-min in 10 mL of lysis buffer in a Falcon tube to facilitate the extraction of proteins (Hurkman and Tanaka, 1986; Murphy and Prinsley, 1985). An aliquot of this well-mixed suspension was diluted with the lysis buffer such that the protein concentration in the diluted mixture was in the range of 0 and 1000 mg L^{-1}. A 0.1 mL portion of this solution was placed in an eppendorf tube and 0.1 mL of SDS solution were added (Dulley and Grieve, 1975; Lees and Paxman, 1972). The resulting mixture was vortexed

(IKA MS 3 basic; IKA Werke GmbH & Co. KG, Staufen, Germany). Reagent C (1 mL) was added to the eppendorf tube. The tube was vortexed and after 10 min, 0.1 mL of Folin reagent were added. This was immediately followed by vortex mixing. After 30 min the absorbance of the sample was measured at a wavelength of 750 nm in a spectrophotometer (UNICAM UV/Vis Spectrometer UV2; Unicam, Cambridge, UK). The spectrophotometer had been zeroed using a blank that had been prepared in exactly the same way as the sample, except that it lacked the protein solution. Interfering substances in the Lowry method have been reviewed by Peterson (1979). Interfering substances generally increase the absorbance of the reagent blank (Peterson, 1979), but this is easily addressed by preparing the blank in exactly the same way as the sample, but without the protein, as in this study. Dilute protein extracts of algal biomass are extremely unlikely to contain interfering substances at concentrations that are above the acceptable threshold (Peterson, 1979) for the Lowry assay.

Sample processing was carried out in the dark to prevent degradation of the Folin reagent. The spectrophotometric absorbance was converted to protein concentration using a calibration curve established with BSA dissolved in lysis buffer. The protein content of the biomass was calculated using the following equation:

$$\text{Protein}\ (\%,\ \text{w/w}) = \frac{CVD}{m} \times 100 \quad (1)$$

where C is the protein concentration (mg L^{-1}) obtained from the calibration curve, V is the volume (L) of the lysis buffer used to resuspend the biomass, D is the dilution factor and m is the amount of biomass (mg).

2.6. Statistical analysis

Four independent measurements were made for each species under each steady state operation. Results were analyzed by means of Microsoft Excel 2003 and Statgraphics 5.1 software. Replicate measurements of protein nitrogen and total nitrogen were reproducible to within ±5% of the respective mean values.

3. Results and discussion

The Lowry method is one of the most accurate methods for quantifying proteins (Peterson, 1979). This method requires comparing the spectrophotometric absorbance of the unknown sample at a wavelength of 750 nm to a calibration curve prepared using standard solutions of a protein. Bovine serum albumin (BSA) is the most commonly used standard. In principle, undiluted protein solutions ranging in concentration from 10 to 1000 mg L^{-1} can be measured directly. In practice, the measurement range should remain confined to the linearity range of the standard calibration curve. Using BSA as the standard, the calibration curve was linear in the absorbance range of 0–0.7 corresponding to a protein concentration range from 0 to 500 mg L^{-1} (Fig. 1). Within this range, the correlation coefficient was >0.997 (Fig. 1).

Intracellular protein can be determined accurately only if all of it is accessible to the reagents used in the Lowry method. The cells must therefore be pretreated to release all the cellular protein. Milling with aluminum oxide for 5-min was previously found to achieve total disruption of algal cells and longer periods of milling did not further increase the concentration of protein in the cell homogenates (Cerón et al., 2008).

P. cruentum was the only species completely lysed with lysis buffer only (Fig. 2) presumably because it lacks a wall and is surrounded only by a thin mucilaginous layer (Arad et al., 1988). *P. cruentum* has indeed been previously reported to be relatively fragile and adversely affected by excessive turbulence in the culture

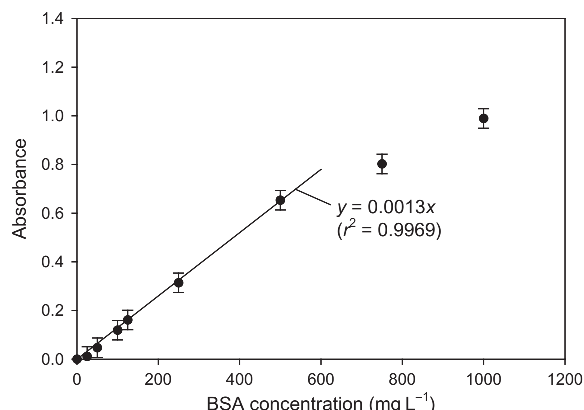

Fig. 1. Absorbance versus protein (BSA) concentration standard curve based on 25 measurements up to a BSA concentration of 500 mg L^{-1}.

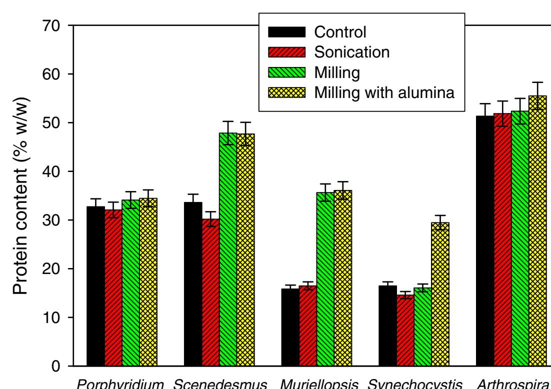

Fig. 2. Total measured protein content of identical biomass samples subjected to different cell disruption pretreatments. Each bar is an average of four replicates.

environment (Mazzuca Sobczuk et al., 2006). Ultrasonication did not enhance protein recovery relative to control (Fig. 2).

Milling without the addition of particles for 5 min completely released the intracellular proteins from all species except the small cyanobacterium *S. aquatilis* (Fig. 2). This bacterium required the inclusion of aluminum oxide in the milling process (Fig. 2). Therefore, the best pretreatment for all species consists of milling 20 mg of the dry biomass with 20 mg of aluminum oxide for 5-min. This pretreatment was used in all subsequent work for quantifying the Lowry protein. With this method, the Lowry protein content of the biomass ranged from 30% to 55% of dry weight (Fig. 2).

For a given algal species, the intracellular concentration of inorganic and organic nitrogen depends on the growth phase (Lourenço et al., 1998). In continuous culture, at steady state, as used in the current study, the specific growth rate is the same as the dilution rate. Although all biomass samples in this study came from steady state operations at a specific growth rate value of 0.03 h^{-1}, or relatively rapid growth that corresponded to a biomass doubling time of 23 h, species-dependent differences in the ratio of total nitrogen to protein nitrogen in the biomass were observed (Fig. 3). In all cases, most of the nitrogen was associated with proteins, but for the two cyanobacteria, the protein nitrogen to total nitrogen ratio was lower than for the three microalgae (Fig. 3). This difference is likely associated with the relatively high proportion of DNA that is a characteristic of prokaryotic cells (Becker, 1994). The cyanobacterium *A. platensis* had the highest nitrogen content of 12.03% and 8.87% (w/w) total nitrogen and protein nitrogen, respectively (Fig. 3). In contrast, the cyanobacterium *S. aquatilis* had low values of 6.76% and 4.80% for total- and protein-nitrogen, respectively

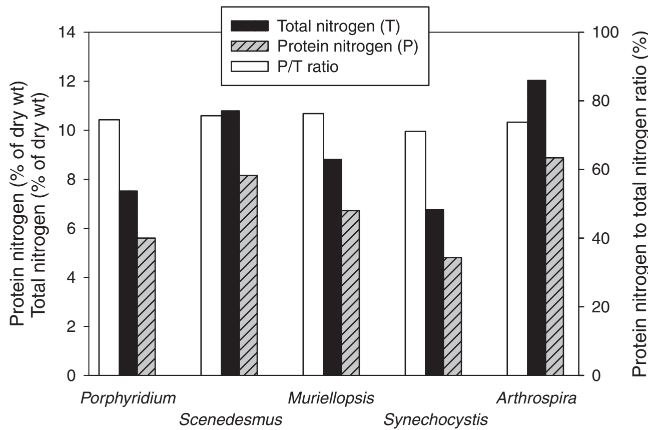

Fig. 3. Total nitrogen and protein nitrogen content of the biomass of various microalgae and cyanobacteria. The ratio of protein nitrogen-to-total nitrogen is also shown. Each bar is an average of four replicates.

(Fig. 3). The nitrogen content for the microalgae were between the extremes seen with the cyanobacteria (Fig. 3).

For the three microalgae species, the protein nitrogen represented 75 ± 1% of the total nitrogen, whereas in the two cyanobacteria the protein nitrogen represented 72 ± 1% of the total nitrogen.

The nitrogen-to-protein conversion factor depended strongly on whether the total nitrogen or Kjeldahl nitrogen was being used for estimating the protein content. For 20 measurements on five diverse microalgae and cyanobacteria undergoing rapid exponential growth, a total nitrogen-to-protein conversion factor of 4.44 was determined with a correlation coefficient of 0.967 (Fig. 4). For all the species, a Kjeldahl nitrogen-to-protein conversion factor of 5.95 was established with a correlation coefficient of 0.944 (Fig. 4). The latter value was significantly different from the Kjeldahl nitrogen-to-protein conversion factor value of 4.78 ± 0.62 recommended by Lourenço et al. (2004) for all algal species, growth conditions or growth phases.

The growth phase also has an impact on the ease of cell disruption, with rapidly growing cells likely to be less robust than slow-growing or stationary-phase cells (Chisti and Moo-Young, 1986). Therefore, milling with ceramic particles is expected to be critically important for ensuring that all the intracellular protein is released from the latter type of cells.

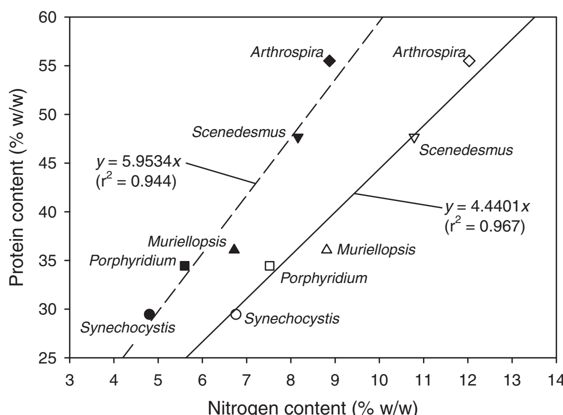

Fig. 4. Correlations between the protein content (Lowry method) of the biomass and: (a) the total measured nitrogen content (hollow symbols); (b) Kjeldahl nitrogen content (filled symbols). The dashed and solid lines are the regression lines through the protein nitrogen and total nitrogen data, respectively.

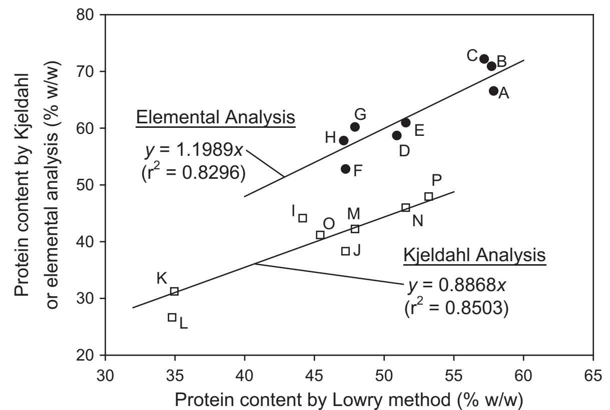

Fig. 5. Correlations between protein content determined by the optimized Lowry method and values obtained from Kjeldahl (hollow squares) and elemental analysis (filled circles) measurements using experimentally determined factors. Samples correspond to different microorganisms: (A) and (B) *S. aquatilis* grown at dilution rate values of 0.04 and 0.01 h^{-1}, respectively; (C) *Anabaena* sp. (ATCC 33047) grown at a dilution rate of 0.05 h^{-1}; (D) *Chlorococcum* sp. (SAG 11.52) grown at a dilution rate of 0.03 h^{-1}; (E) *S. almeriensis* grown at a dilution rate of 0.043 h^{-1} in the specified medium but without nitrogen; (F) *S. almeriensis* grown at a dilution rate of 0.01 h^{-1} in the specified nitrogen sufficient medium; (G) *A. platensis* grown at a dilution rate of 0.02 h^{-1} in the specified culture medium supplemented with carbonate and bicarbonate to a total concentration of 3.6 g C L^{-1}; (H) *A. platensis* grown at a dilution rate of 0.02 h^{-1} in the specified culture medium supplemented with carbonate and bicarbonate to a total concentration of 1.2 g C L^{-1}. (I)–(P) *S. almeriensis* grown at a: dilution rate of 0.017 h^{-1} (I); dilution rate of 0.01 h^{-1} (J); dilution rate of 0.017 h^{-1} in N-deficient medium (K); dilution rate of 0.011 h^{-1} in N-deficient medium (L); dilution rate of 0.032 h^{-1} in the specified N-sufficient medium (M); dilution rate of 0.043 h^{-1} in N-deficient medium (N); dilution rate of 0.029 h^{-1} in N-deficient medium (O); and a dilution rate of 0.042 h^{-1} in N-deficient medium (P). Except for the growth conditions mentioned here, the other conditions were the same as in Section 2. Each regression line is based on 32 measurements.

Fig. 5 compares the Lowry protein content with the protein levels measured using the Kjeldahl method and elemental analysis of randomly selected samples obtained under various growth conditions and pretreated with the optimized procedure. The expected value of the slopes of the two regression lines in Fig. 5 is unity, but in practice neither of the methods agrees exactly with the Lowry measurements. The slopes of the regression lines are within ±20% of the expected value. For 32 measurements spanning eight combinations of species and growth conditions, the correlation coefficients of the two lines are similar at 0.84 ± 0.01. The elemental analysis significantly overestimates the protein contents in comparison to the Lowry method, whereas the Kjeldahl method underestimates it. Nevertheless, the Kjeldahl method more closely correlates to the Lowry protein measurements (Fig. 5) and is a more reliable indicator of the true protein content than the total nitrogen value (elemental analysis).

4. Conclusions

For microalgae and cyanobacteria undergoing rapid growth, the recommended Kjeldahl nitrogen-to-protein conversion factor is 5.95 instead of 6.25. If total nitrogen is measured, a conversion factor value of 4.44 should be used to estimate the protein content. Kjeldahl nitrogen value better correlates with the Lowry protein measurement than does the total nitrogen value. Total protein content in the biomass depends strongly on the microbial species. For the species assessed in this work, the total protein content during rapid steady state growth ranged from 30% to 55% of dry weight. Milling the biomass with ceramic particles is essential for completely releasing the intracellular protein prior to measurement by the Lowry method.

Acknowledgements

This work was supported by Ministerio de Educación y Ciencia (CTQ2004-07628-C02-01/PPQ), CENIT-CO2 project with financing by ENDESA S.A., Ministerio de Industria (CDTI), Junta de Andalucía and Plan Andaluz de Investigación (BIO 173), Spain.

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