Glasgow Caledonian University

University for the Common Good

Optimization of cell disruption in Raphidocelis subcapitata and Chlorella vulgaris for biomarker evaluation

Aderemi, Adeolu; Hunter, Colin; Pahl, Ole; Shu, Xinhua

Published in:
Journal of microbiology, biotechnology and food sciences

DOI:
10.15414/jmbfs.2015.4.6.481-484

Publication date:
2015

Document Version
Publisher's PDF, also known as Version of record

Link to publication in ResearchOnline

Citation for published version (Harvard):
Aderemi, A, Hunter, C, Pahl, O & Shu, X 2015, 'Optimization of cell disruption in Raphidocelis subcapitata and Chlorella vulgaris for biomarker evaluation', *Journal of microbiology, biotechnology and food sciences*, vol. 4, no. 6, pp. 481-484. https://doi.org/10.15414/jmbfs.2015.4.6.481-484

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
If you believe that this document breaches copyright please view our takedown policy at https://edshare.gcu.ac.uk/id/eprint/5179 for details of how to contact us.

Download date: 12. Oct. 2026



JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

OPTIMIZATION OF CELL DISRUPTION IN RAPHIDOCELIS SUBCAPITATA AND CHLORELLA VULGARIS FOR BIOMARKER EVALUATION

Adeolu Aderemi[1], Colin Hunter[1], Ole Pahl[1], Xinhua Shu[2]*

Address(es):
[1]Glasgow Caledonian University, School of Engineering and Built Environment, 70 Cowcaddens Road, G4 0BA, Glasgow, UK.
[2]Glasgow Caledonian University, Department of Life Sciences, 70 Cowcaddens Road, G4 0BA, Glasgow, UK.

*Corresponding author: Adeolu.Aderemi@gcu.ac.uk

doi: 10.15414/jmbfs.2015.4.6.481-484

ARTICLE INFO

Received 12. 9. 2014
Revised 24. 2. 2015
Accepted 5. 3. 2015
Published 1. 6. 2015

Regular article

OPEN ACCESS

ABSTRACT

Raphidocelis subcapitata and *Chlorella vulgaris* are bioassay microalgae with rigid cellulosic cell wall which can hinder the release of intracellular proteins often studied as toxicity biomarkers. Since cell disruption is necessary for recovering intracellular biomolecules in these organisms, this study investigated the efficiency of ultrasonication bath; ultrasonication probe; vortexer; and bead mill in disintegrating the microalgae for anti-oxidative enzyme extraction. The extent of cell disruption was evaluated and quantified using bright field microscopy. Disrupted algae appeared as ghosts. The greatest disintegration of the microalgae (83-99.6 %) was achieved using bead mill with 0.42-0.6 mm glass beads while the other methods induced little or no disruption. The degree of cell disruption using bead mill increased with exposure time, beads-solution ratio and agitation speed while larger beads caused less disruption. Findings revealed that bead milling, with specific parameters optimized, is one of the most effective methods of disintegrating the robust algal cells.

Keywords: Microalgae; cell disruption; ghost cells; ultrasonication; bead beating; biomarker

INTRODUCTION

The use of the unicellular microalgae, *Raphidocelis subcapitata* and *Chlorella vulgaris* as bioassay test organisms is significant as they play a vital role in the structure and functioning of the aquatic ecosystems and any adverse effect on them will affect the upper trophic levels (**Li *et al.*, 2006**). In ecotoxicological studies, biomolecules such as the antioxidative enzymes and heat stress proteins are often used as biomarkers in assessing the adverse effects of various environmental toxicants on aquatic organisms (**Dewez *et al.*, 2005; Bierkens *et al.*, 1998**). Protocols for determining the activities of some of these biomolecules in test organisms usually involve an initial cell disruption or homogenization step. In microalgae, cell disintegration is often necessary for the recovery of intracellular products (**Mendes-Pinto *et al.*, 2002; Molina Grima *et al.*, 2003**). However, the cell walls of certain phytoplankton such as *C. vulgaris* and *R. subcapitata* are composed predominantly of hemicellulose and saccharides which hinder the release of intracellular biological molecules (**Abo-Shady *et al.*, 1993; Doucha and Livansky, 2008**). Since membrane and/or cell wall species-specific composition could have an effect on disruption efficiency in different organisms, it is expected that the extent of cell disintegration will depend on the algal species (**Lavoie *et al.*, 2009**).

A range of treatment methods have been suggested and used for breaking algal cells. These include freezing, alkali and organic solvents, sonication, high pressure homogenization (French press), bead milling, microwaving, enzymatic lysis and manual grinding (**Wimpenny, 1967; Chisti and Moo-young, 1986; Molina Grima *et al.*, 2004; Zheng *et al.*, 2011**). *Chlorococcum sp*., *Botryococcus sp*., *C. vulgaris*, *R. subcapitata* and *Chlamydomonas reinhardtii* have been lysed for various purposes employing different techniques such as high pressure homogenization, sulphuric acid treatment, bead beating, ultrasonics, microwaves, liquid nitrogen grinding, rotor stator homogenizer and vortex agitator (**Halim *et al.*, 2012; Lee *et al.*, 2010; McMillan *et al.*, 2013; Zheng *et al.*, 2011; Lavoie *et al.*, 2009**). The French press, sonication and bead beating are considered the most effective and widely used methods for the homogenization of single cells, cell suspensions and microorganisms for the recovery of intracellular products (**Burden, 2012**).

During ultrasonication process, high frequency sonic waves created by the expansion and contraction of a crystallized probe or bath, bring about implosive collapse of the gas-filled cavitation bubbles and generate intense microscopic shock waves, which ultimately cause cell wall disruption (**Zheng *et al.*, 2011**).

Bead beating is a simple disruption technique that breaks cells by shaking a closed container filled with the target cells and beads made up of quartz or metal (**Kim *et al.*, 2013**). It uses not only beads but other grinding media such as balls and satellites, while non-spherical media such as garnet has been effectively used in slicing resilient samples when used in conjunction with larger grinding balls (**Burden, 2012**). Although, some of these homogenization techniques are relatively old and are frequently used (**Lavoie *et al.*, 2009**), information in literature about their efficiency in breaking the resilient cell wall of *R. subcapitata* and *C. vulgaris* as well as the endpoint of cell disruption is scarce.

The aim of this study was to assess the efficacy of four mechanical treatment methods in breaking *R. subcapitata* and *C. vulgaris* cells for antioxidant enzymological study. These methods were ultrasonication bath, ultrasonication probe, vortex agitation and bead milling. The degree of algal cell disruption was evaluated and quantified using direct optical microscopy techniques.

MATERIAL AND METHODS

Strain and Cultivation

The microalgae, *R. subcapitata* formerly known as *Selenastrum capricornutum* (strain CCAP 278/4) and *C. vulgaris* (strain CCAP 211/12) were obtained from Culture Collection of Algae and Protozoa (CCAP), UK, and grown in 500-ml Erlenmeyer flasks containing sterile Jaworski's medium (JM). The cultures were maintained on a shaker (100 rpm) in a culturing chamber at $20^0C \pm 1^0C$ under continuous irradiance in the range 2000-3000 lux. Algal cells (10^4 cells/ml) were transferred after 5 days to fresh JM in 50-ml conical flasks and cultivated for 5 days under the same environmental conditions. Algal cells were then harvested in sodium phosphate buffer (pH 7) and treated with either. : ultrasonic bath, ultrasonic probe, vortex agitation and bead beating for varying times before being microscopically analysed for cell rupture. Control samples were without treatment and each treatment was done in triplicate.

Methods for cell disruption of algal cells

Ultrasonic bath

Sonication was applied indirectly to algae suspension (5.2 - 5.4 x 10^6 cells/ml) inside glass tubes in a 75 W ultrasonic bath (S $_{30}$, D-78224 Singen/Htw, Elma

Ltd, Germany) with an ultrasonic wave frequency of 50/60 KHz. The samples were treated for 60 min and 30µl samples were taken for analysis at 15 min intervals. Since the distribution of ultrasonic intensity in an ultrasonic bath is not homogenous, the aluminium foil test **(Mason, 2000**) was performed prior to the sample treatment to locate the position with the highest sonication intensity.

Ultrasonic probe

Direct sonication was applied to cell suspensions (5.0 - 5.5 x 10^6 cells/ml) in eppendorf tubes with an ultrasonic processor (Soniprep 150, MSE, UK) equipped with 3 mm exponential tip, at 100% amplitude giving 20 KHz ultrasonic wave frequency for 2, 5, and 10 min. The samples were kept on ice during the sonication process to avoid overheating.

Sonication/Repeated freezing and thawing (RFT)

Cell suspensions sonicated using the above methods underwent slow freezing at -20^0C after which they were flash frozen in liquid nitrogen followed by slow thawing in ice bath. Three separate trials of freezing/thawing cycles were conducted. 30µl samples of the algae were taken after each freezing-thawing cycle for microscopic analysis.

Vortex agitation

Samples (6.0 – 6.4 x 10^5 cells/ml) were agitated in eppendorf tubes using 0.56-0.7 mm garnet sand in combination with two 6.35 mm zirconium balls (lysing matrix A, QBiogene, USA) using a vortex mixer (VB3B011, SciQuip Ltd., UK) at 2500 rpm for 2 and 5 min processing time.

Bead mill homogenizer

Algal samples (6.4 – 7.0 x 10^5cells/ml) were ground in 1.5 ml tubes containing 0.42-0.60 mm glass beads (Sigma) or 1.0 mm silica spheres (lysing matrix C, MP Biomedicals) or 3.15 mm stainless steel balls (lysing matrix S, MP Biomedicals) with a beads/solution ratio of 50%vol/vol and 70%vol/vol for 1, 3 and 5 min using a shaking type multitube bead beater (FastPrep®-24, MP Biomedicals, France) at 4.5 and 6.5 ms^{-1}.

Quantifying and evaluating cell disruption

In order to quantify the effectiveness of each treatment, the fraction of physically disrupted cells were measured. This was achieved by counting the number of intact cells treated after a specific time interval against those initially determined from the control values **(McMillan *et al.*, 2013**). A Leica DM500 microscope was employed in the bright field configuration moving between 10 × and 40 × objectives. The microscope was equipped with a digital camera (Leica ICC50, Leica UK Ltd) linked to a desktop computer using Leica software (LAS EZ, Switzerland) where images were grabbed and stored. 30 µl of the treated algae sample or control was pipetted onto a slide for examination under the microscope. 10 µl of the sample was also pipetted onto the loading area of a haemocytometer and covered with a cover slip, for counting under the microscope (40 x). At each position, the focus was adjusted and the intact cells counted. D_t, the total cell disruption at time t, was quantified in terms of the ratio of the average number of intact cells counted after a treatment time (I_t) to the initial counts of intact cells at t = 0, denoted I_0. D_t then, is given by

$$D_t = (1- I_t / I_0) \times 100 \quad (1)$$

where the ratio I_t/I_0 indicates the cell survival probability (**McMillan *et al.*, 2013**).

RESULTS AND DISCUSSION

Microscopy and Disruption Methods

The effects of the different treatments on cell disruption efficiency are shown in Figure 1 while a view of the suspensions of *Raphidocelis* and *Chlorella* cells before and after treatment is given in Figure 2. The algae species were observed to be intact before treatment (Fig. 2a and 2b) while damaged or disrupted *R. subcapitata* and *C. vulgaris* appeared as empty or ghost cells following bead milling (Fig. 2c and 2d respectively). Ghost cells are dead cells with visible outlines devoid of nucleus and cytoplasmic components (**Stedman, 1995**). The observation of damaged microalgae, *Isochryis galbana*, *Pavlova sp.*, *Tetraselmis striata*, *Nannochloropsis sp.* and *Chlorella sp.* as ghost cells following long periods of refrigerated storage has been reported in literature (**Espinosa and Allam, 2006**). There was no significant difference in the degree of cell disintegration induced by the disruption methods between the phytoplankton species ($p > 0.05$). The disruption efficiency of the treatments in *R. subcapitata* and *C. vulgaris* are as follows: ultrasonic bath (0, 0%); ultrasonic probe (2.3, 2.6%); vortex agitation (0.9, 0.5%) and bead beating (99.6, 99.2%) respectively. Indirect sonication using a bath had no disruptive effects on the microalgae with the cells remaining intact after an hour of treatment. This may be attributed to the heating produced in ultrasonic baths which can be transmitted to the samples, interfering with sonication intensity or cavitation (**Patricio *et al.*, 2006**).

Surprisingly, liquid shearing with a sonic probe was hardly effective in breaking the algal cells, resulting in less than 3 % disruption of the algae species. Ultrasonic probe delivers about 100 times the ultrasonic intensity of an ultrasonic bath (**Santos *et al.*, 2008**) and its use for rapid algal cell homogenization has been reported in literature (**Bierkens *et al.*, 1998; Zargar *et al.*, 2006**). **Lavoie *et al.* (2009)** achieved 26.8 % and 98 % disruption efficiency in *R. subcapitata* and *C. reinhardtii* respectively using sonication and it was the least effective method in breaking *Botryococcus* cells at 8.8 % (**Lee *et al.*, 2010**). Its ineffectiveness is likely due to the differences in the equipment and size of the tip used. The probe or detachable horn is the most important part of a sonicator and its shape or design has an influence on sonication intensity or performance (**Santos *et al.*, 2008**). The stepped probe is more suitable for micro-applications and transmits a much higher ultrasonic energy than the exponential tip (**Santos *et al.*, 2008**). Slow freezing followed by rapid freezing of biological samples is known to induce intracellular ice crystals, and this can lead to the osmotic rupturing of cells on thawing due to the presence of water (**Nord and Bier, 1952**). However, RFT had no effects on disruption or sonication efficiency in this study, in consonance with previous report by **Lavoie *et al.* (2009)**.

Vortex agitation yielded very poor results with over 99% of cells presenting intact walls after 10 min of agitation. Vortexers are readily available and as a result, have been adapted to bead beat biological samples. However, they lack the power of the true bead beaters, thus they are less effective at cell disruption (**Burden, 2012**). About 18 % and 99 % disruption of *R. subcapitata* and *C. reinhardtii* cells respectively were obtained using a vortex mixer containing 0.5 mm zirconia-silica beads with beads/solution ratio of 90 % vol/vol and an agitation time of 6 min (**Lavoie *et al.*, 2009**). Vortexing with quartz sand for 3 min was also found to be adequate in the disruption of *D. subspicatus* (**Tukaj and Tukaj, 2010**). Its inefficiency in this study possibly lies in the differences in grinding media as well as the mechanical strength of algal cell walls.

Bead Milling

Bead beating with 0.42-0.6 mm glass beads was the most effective treatment method in this study disagreeing with some previous studies that showed bead beating was not as efficient as other approaches (**Cheng *et al.*, 2010; Prabakaran and Ravindran, 2011; Sheng *et al.*, 2012; Zheng *et al.*, 2011**). It resulted in 83-99.6 % disruption of the algal cells as evidenced by the ghost cells, developing ghosts, and cell debris produced within 1-5 min of treatment. **Lee *et al.* (2010)** found bead beating to be the most effective method of disintegrating *Botryococcus sp.* for lipid extraction (28.1 %) while more than 90 % of *Chlorella* cells were disrupted when passed through different bead mills using glass and zirconium beads in the range 0.3-0.7 mm (**Doucha and Livansky, 2008**). Bead beating was also found to extract the highest lipid content from wet pellets of *Botryococcus braunii* when compared to ultrasonication and high pressure homogenization (**Lee *et al.*, 1998**).

The effects of the beads diameter and the agitation speed on the degree of algal cell breakage were studied (Tables 1 & 2). Using beads-solution ratio of 50%vol/vol and maximum speed of 6.5 ms^{-1}, the glass beads (0.42-0.6 mm) caused the highest disruption (99.3 % and 99 %) of *Raphidocelis* and *Chlorella* cells respectively. Disintegration of the algae species was found to decrease with increase in beads diameter with the larger 1 mm silica beads and 3.15 mm stainless steel balls inducing 56 %, 49 % and 16 %, 19 % disruption respectively in *R. subcapitata* and *C. vulgaris*. Less disruption (87 %, 84 %) of *Raphidocelis* and *Chlorella* cells respectively was obtained with the glass beads at lower agitation speed of 4.5 ms^{-1}. The impact of disintegration time and the volume of beads to cell suspension ratio on the extent of cell disruption in the algal cells was also investigated (Tables 3 & 4 respectively). The disruption efficacy of the algal cells using a bead size 0.42-0.6 mm was found to increase with beads filling and exposure time reaching a peak after 5 min. The lowest disruption (83 %, 85.5 %) was obtained using 50% vol/vol bead-solution ratio within 60 seconds exposure time for *Chlorella* and *Raphidocelis* cells respectively. The highest disruption resulted after 5 min of processing time with 50 %vol/vol and 70%vol/vol beads loading causing (99.3 %, 99 %) and (99.6 %, 99.2 %) disruption of *Raphidocelis* and *Chlorella* cells respectively.

According to **Hopkins (2014)**, the beads size and the volume ratio of beads to cell suspension are influential on the degree of cell disintegration. About 90 % of *Scenedesmus obliquus* were disrupted in a bead mill using 0.35-0.50 mm glass beads as compared to 0.5-0.7 mm beads (80 % disruption) and 1.1-1.2 mm beads (50 % disruption) (**Hedenskog *et al.*, 1969**). More *Raphidocelis* and *Chlorella* cells were disrupted when the speed of agitation was increased in this study. The impacts of these parameters including agitation speed on the disruption efficiency of bead mills were earlier investigated by **Doucha and Livansky (2008)**, achieving 99% disintegration of *Chlorella* cells with optimum glass bead diameter of 0.42-0.58 mm, 82 % beads filling and agitator speed of 14ms^{-1} using a Dyno-Mill KDL-Pilot.

The discrepancies among some of the published reports on the efficiency of bead mill in algal cell disintegration may be attributed to various factors such as variations in container shape, agitation speed, bead size, bead composition and

bead loading (**Kim *et al.*, 2013**). According to Kim et al. (2013), the advantages of this technique lie in its simplicity and high throughput. However, there is the need to bead beat for short time intervals with cooling on ice between each interval in order to control the thermal degradation of the target enzymes or proteins.

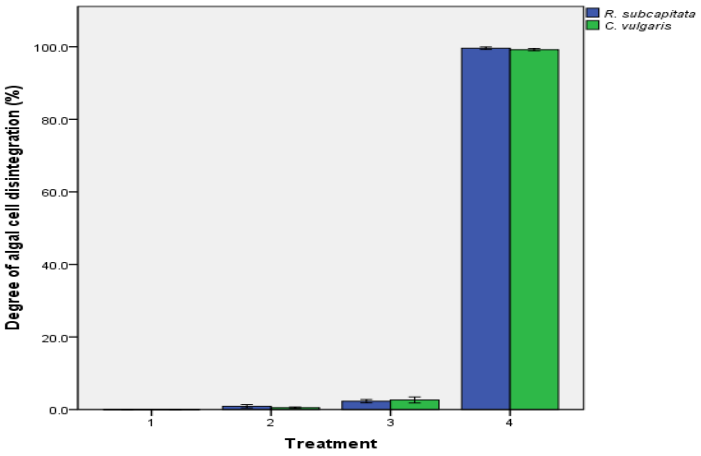


Figure 1 Effects of different methods on cell disruption of *R. subcapitata* and *C. vulgaris*. *1* Sonication bath, *2* vortex agitation, *3* sonication probe, *4* bead mill (6.5 m/s, 5 min and 70 % beads filling), Error bars represent standard error of mean

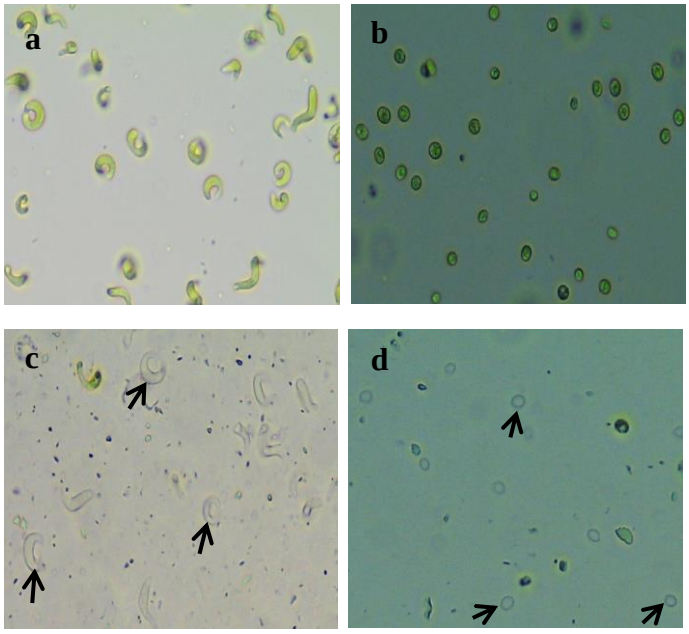


Figure 2 Suspension of *Raphidocelis* cells before (a) and after (c) treatment (96 % disruption efficiency) and *Chlorella* cells before (b) and after (d) treatment (93 % disruption efficiency), (FastPrep®-24 homogenizer, glass beads 0.42-0.6 mm, agitation speed 6.5 ms^{-1}, 3 min processing time, arrows showing ghost cells).

Table 1 Effects of bead diameter and agitation speed on the degree of *R. subcapitata* cell disintegration in the bead mill after 5 min

Trial no	Beads filling (%)	Agitation speed (ms^{-1})	Degree of cell disruption (%)*
Glass beads 0.42-0.6 mm			
1	50	4.5	87 ± 0.86
2	50	6.5	99.3 ± 0.45
Silica spheres 1.0 mm			
1	50	6.5	56 ± 4.1
Stainless steel balls 3.15 mm			
1	50	6.5	16 ± 2.0

*Values represent mean of three replicate assays ± standard error of mean (SEM)

Table 2 Effects of bead diameter and agitation speed on the degree of *C. vulgaris* cell disintegration in the bead mill after 5 min

Trial no	Beads filling (%)	Agitation speed (ms^{-1})	Degree of cell disruption (%)*
Glass beads 0.42-0.6 mm			
1	50	4.5	84 ± 1.5
2	50	6.5	99 ± 0.2
Silica spheres 1.0 mm			
1	50	6.5	49 ± 4.3
Stainless steel balls 3.15 mm			
1	50	6.5	19 ± 1.5

*Values represent mean of three replicate assays ± standard error of mean (SEM)

Table 3 Influence of disintegration time and beads filling on the degree of *R. subcapitata* cell disintegration in the bead mill at maximum speed of 6.5 ms^{-1}

Trial no	Beads filling (%)	Disruption time (min)	Degree of cell disruption (%)*
Glass beads 0.42-0.6 mm			
1	50	1	85.5 ± 4.5
2	70	1	89 ± 3.6
3	50	3	96 ± 2.5
4	70	3	98 ± 0.5
5	50	5	99.3 ± 0.45
6	70	5	99.6 ± 0.1

*Values represent mean of three replicate assays ± standard error of mean (SEM)

Table 4 Influence of disintegration time and beads filling on the degree of *C. vulgaris* cell disintegration in the bead mill at maximum speed of 6.5 ms^{-1}

Trial no	Beads filling (%)	Disruption time (min)	Degree of cell disruption (%)*
Glass beads 0.42-0.6 mm			
1	50	1	83 ± 2.1
2	70	1	87 ± 1.5
3	50	3	93 ± 2.6
4	70	3	98 ± 0.76
5	50	5	99 ± 0.2
6	70	5	99.2 ± 0.1

*Values represent mean of three replicate assays ± standard error of mean (SEM)

CONCLUSION

In addition to identifying ghost cells as indicators of cell disruption, the findings from this study clearly revealed that bead milling, using the appropriate parameters, is one of the most effective methods in disrupting microalgae species including the resilient *C. vulgaris* and *R. subcapitata* cells for the recovery of intracellular products.

Acknowledgments: This work was partly funded by the EU Transnational Territorial Cooperation programme INTERREG IVB NWE projects (PILLS project 008B & "noPILLS in our waters"); this support is greatly acknowledged.

REFERENCES

ABO-SHADY, A.M., MOHAMED, Y.A., LASHEEN, T. 1993. Chemical composition of the cell wall in some green algae species. *Biol Plantarum* 35(4), 629-632. http://dx.doi.org/10.1007/bf02928041

BIERKENS, J., MAES, J., PLAETSE, F.V. 1998. Dose-dependent induction of heat shock protein 70 synthesis in *Raphidocelis subcapitata* following exposure to different classes of environmental pollutants. *Environ Pollut* 101, 91-97. http://dx.doi.org/10.1016/s0269-7491(98)00010-4

BURDEN, D.W. 2012. Guide to the disruption of biological samples. Random Primers, OPS Diagnostics. http://opsdiagnostics.com/applications/opsd_2012_disruption_guide_v1.1.pdf. Accessed 29 October 2013

CHENG, Y.L., JUANG, Y.C., LIAO, G.Y., HO, S.H., YEH, K.L., CHEN, C.Y., CHANG, J.S., LIU, J.C., LEE, D.J. 2010. Dispersed ozone flotation of *Chlorella vulgaris*. *Bioresour Technol* 101, 9092–9096. http://dx.doi.org/10.1016/j.biortech.2010.07.016

CHISTI, Y., MOO-YOUNG, M. 1986. Disruption of microbial cells for intracellular products. *Enzyme Microb Technol* 8, 194–204. http://dx.doi.org/10.1016/0141-0229(86)90087-6

DEWEZ, D., GEOFFROY, L., VERNET, G., POPOVIC, R. 2005. Determination of photosynthetic and enzymatic biomarkers sensitivity used to evaluate toxic effects of copper and fludioxonil in alga *Scenedesmus obliquus*. *Aquat Toxicol* 74, 150-159. http://dx.doi.org/10.1016/j.aquatox.2005.05.007

DOUCHA, J., LIVANSKY, K. 2008. Influence of processing parameters on disintegration of *Chlorella* cells in various types of homogenizers. *Appl Microbiol Biotechnol* 81, 431-440. http://dx.doi.org/10.1007/s00253-008-1660-6

ESPINOSA, E.P., ALLAM, B. 2006. Comparative growth and survival of juvenile hard clams, *Mercenaria mercenaria* fed commercially available diets. *Zoo Biol* 25(6), 513-525. http://dx.doi.org/10.1002/zoo.20113

HALIM, R., HARUN, R., DANQUAH, M., WEBLEY, P. 2012. Microalgal cell disruption for biofuel development. *Appl Energy* 91, 116-121. http://dx.doi.org/10.1016/j.apenergy.2011.08.048

HEDENSKOG, G., ENEBO, L., VENDLOVÁ, J., PROKEŠ, B. 1969. Investigation of some methods for increasing the digestibility in vitro of microalgae. *Biotechnol Bioeng* 11, 37–51. http://dx.doi.org/10.1002/bit.260110104

HOPKINS, T. 2014. Cell disrupters: A review covering apparatus and techniques of cell disruption. Biospec. http://www.biospec.com/laboratory_cell_disrupters/. Accessed 23 March 2014.

KIM, J., YOO, G., LEE, H., LIM, J., KIM, K., KIM, C.W., PARK, M.S., YANG, J.W. 2013. Methods of downstream processing for the production of biodiesel from microalgae. *Biotechnol Advances* 31(6), 862-876. http://dx.doi.org/10.1016/j.biotechadv.2013.04.006

LAVOIE, M., BERNIER, J., FORTIN, C., CAMPBELL, P.G.C. 2009. Cell homogenization and subcellular fractionation in two phytoplanktonic algae: implications for the assessment of metal subcellular distributions. *Limnol Oceanogr: methods* 7, 277-286. http://dx.doi.org/10.4319/lom.2009.7.277

LEE, S.J., KIM, S.B., KIM, J.E., KWON, G.S., YOON, B.D., OH, H.M. 1998. Effects of harvesting method and growth stage on the flocculation of the green alga *Botryococcus braunii*. *Lett Appl Microbiol* 27, 14–18. http://dx.doi.org/10.1046/j.1472-765x.1998.00375.x

LEE, S.J., YOO, C., JUN, S.Y., AHN, C.Y., OH, H.M. 2010. Comparison of several methods for effective lipid extraction from microalgae. *Bioresour Technol* 101, S75-S77. http://dx.doi.org/10.1016/j.biortech.2009.03.058

LI, M., HU, C., ZHU, Q., CHEN, L., KONG, A., LIU, Z. 2006. Copper and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities in the microalga *Pavlova viridis* (Prymnesiophyceae). *Chemosphere* 62, 565-572. http://dx.doi.org/10.1016/j.chemosphere.2005.06.029

MASON, T.J. 2000. Sonochemistry. Oxford chemistry primers, Oxford, UK, 96p. ISBN 978-0198503712

MCMILLAN, J.R., WATSON, I.A., ALI, M., JAAFAR, W. 2013. Evaluation and comparison of algal cell disruption methods: microwave, waterbath, blender, ultrasonic and laser treatment. *Appl Energy* 103, 128-134. http://dx.doi.org/10.1016/j.apenergy.2012.09.020

MENDES-PINTO, M.M., RAPOVO, M.F.J., BOWEN, J., ZOUNY, A.J., MORAIS, R. 2001. Evaluation of different cell disruption processes on encysted cells of *Haematococcus pluvialis*: effects on astaxanthin recovery and implications for bio-availability. *J Appl Phycol* 13, 19-24. http://dx.doi.org/10.1023/a:1008183429747

MOLINA GRIMA, E., ACIÉN FERNÁNDEZ, F.G., ROBLES MEDINA, A. 2004. Downstream processing of cell-mass and products. In: RICHMOND A (ed) handbook of microalgal culture, *Biotechnol Appl Phycol*, Blackwell, 215–252. http://dx.doi.org/10.1002/9780470995280.ch10

MOLINA GRIMA, E., BELARBI, E.H., ACIEN FERNANDEZ F.G., ROBLES MEDINA, A., VHOSTI, Y. 2003. Recovery of microalgal biomass and metabolites: process options and economics. *Biotechnol Advances* 20, 491-515. http://dx.doi.org/10.1016/s0734-9750(02)00050-2

NORD, F.F., BIER, M. 1952. In: PLANK (ed) hanbuch der kaltetechnik, Springer-Verlag, Heidelberg, 9, 84-166. http://dx.doi.org/10.1007/978-3-642-94595-3_2

PATRICIO, A., FERNANDEZ, C., MOTA, A.M., CAPELO, J.L. 2006. Dynamic versus static ultrasonic sample treatment for the solid-liquid pre-concentration of mercury from human urine. *Talanta* 69(3), 769-775. http://dx.doi.org/10.1016/j.talanta.2005.11.007

PRABAKARAN, P., RAVINDRAN, A.D. 2011. A comparative study on effective cell disruption methods for lipid extraction from microalgae. *Lett Appl Microbiol* 53(2), 150–154. http://dx.doi.org/10.1111/j.1472-765x.2011.03082.x

SANTOS, H.M., LODEIRO, C., CAPELO, J.L. 2008. The power of ultrasound. in: CAPELO JL (ed) ultrasound in chemistry: analytical applications, Wiley-vch Verlag Gmbh & co. KGaa, Weinheim. http://dx.doi.org/10.1002/9783527623501.ch1

SHENG, J., VANNELA, R., RITTMANN, B.E. 2012. Disruption of *Synechocystis* PCC 6803 for lipid extraction. *Water Sci Technol* 65(3), 567–573. http://dx.doi.org/10.2166/wst.2012.879

STEDMAN, T. 1995. Ghost cell In: the American heritage® Stedman's medical dictionary. Houghton Mifflin Company, 923p. ISBN 03956995X. http://dictionary.reference.com/browse/ghost cell. Accessed 15 July 2014.

TUKAJ, S., TUKAJ, Z. 2010. Distinct chemical contaminants induce the synthesis of Hsp70 proteins in green microalgae *Desmodesmus subspicatus*: heat pretreatment increases cadmium resistance. *J Therm Biol* 35, 239-244. http://dx.doi.org/10.1016/j.jtherbio.2010.05.007

WIMPENNY, J.W.T. 1967 Breakage of microorganisms. *Proc Biochem* 2, 41–44.

ZARGAR, S., KRISHNAMURTHI, K., DEVI, S., GHOSH, T.K., CHAKRABARTI, T. 2006. Temperature-induced stress on growth and expression of Hsp in freshwater alga *Scenedesmus quadricauda*. *Biomed Environ Sci* 19, 414-421.

ZHENG, H., YIN, J., GAO, Z., HUANG, H., JI, X., DOU, C. 2011. Disruption of *Chlorella vulgaris* cells for the release of biodiesel-producing lipids: a comparison of grinding, ultrasonication, bead milling, enzymatic lysis, and microwaves. *Appl Biochem Biotechnol* 164: 1215-1224. http://dx.doi.org/10.1007/s12010-011-9207-1