Research article **Open Access**

Y. Xu, J.J. Milledge, A. Abubakar, R.A.R. Swamy, D. Bailey, P.J. Harvey*

Effects of centrifugal stress on cell disruption and glycerol leakage from *Dunaliella salina*

DOI 10.1515/micbi-2015-0003
Received October 6, 2014; accepted May 5, 2015

Abstract: *Dunaliella salina* accumulates large amounts of intracellular glycerol in response to the increases in salt concentration, thus is a potential source for producing fuel grade glycerol as an alternative to biodiesel-derived crude glycerol. *D. salina* lacks a cell wall; therefore the mode of harvesting *Dunaliella* cells is critical to avoid cell disruption caused by extreme engineering conditions. This study explored cell disruption and glycerol leakage of *D. salina* under various centrifugal stresses during cell harvesting. Results show a centrifugal g-force lower than 5000 g caused little cell disruption, while a g-force higher than 9000 g led to ~40% loss of the intact cells and glycerol yields from the recovered algal pellets. Theoretical calculations of the centrifugal stresses that could rupture *Dunaliell*a cells were in agreement with the experimental results, indicating optimisation of centrifugation conditions is important for recovering intact cells of *D. salina* enriched in glycerol.

Keywords: *Dunaliella salina*; microalgae; glycerol; centrifugation; disruption; harvesting

Abbreviations

English letters used in equations

Symbol	Description	Unit
d_c	Cell diameter	m
F_d	Viscous drag	N
g_n	Acceleration due to gravity (9.81)	m s^{-2}
g	g-force a multiple of the standard acceleration due to gravity (xg)	
g_c	The product of g and g_n	m s^{-2}
h	Liquid height	m
M	Molecular mass in Daltons	Da
p	Pressure	Pa
p_h	Hydrostatic pressure	Pa
r_c	Cell radius	m
r_p	Protein radius	m
R_e	Reynolds Number	
t	Thickness of the wall or membrane	m
v	Relative fluid velocity	m s^{-1}

Greek symbols used in equations

Symbol	Description	Unit
μ	Viscosity	Pa s
π	Pi	
ρ	Density	kg m^{-3}
ρ_s	Density of solid	kg m^{-3}
ρ_l	Density of liquid	kg m^{-3}
σ_y	Yield stress	Pa

1 Introduction

Microalgae have been playing an ever increasingly important role in biotechnology industry because of their natural abundance of valuable products, such as lipids, fatty acids, proteins, pigments and bioactive compounds [1]. Algal biorefineries typically integrate biomass conversion processes and equipment with the aim of taking advantage of all the components in the raw materials to achieve overall economic production of, typically, fuels, power and chemicals [2,3]. The commercial utilisation of green microalga *Dunaliella salina* is ideally suited for demonstrating the principles of an algal biorefinery. Representing the largest (100s ha) of current commercial cultivation technologies for any microalga [1,4], *D. salina* is currently cultivated commercially in highly saline non-potable waters for β-carotene production, the content of which in *D. salina* vastly exceeds that found in many land plants [5]. However, β-carotene is not the only compound of commercial interest: *D. salina* cells also produce a wide range of other carotenoids, oxycarotenoids, lipids, proteins and other compounds of commercial value [4].

***Corresponding author: P.J. Harvey:** University of Greenwich, School of Science, Central Avenue Chatham Maritime, Kent ME4 4TB, E-mail: p.j.harvey@gre.ac.uk
Y. Xu, J.J. Milledge, A. Abubakar, R.A.R. Swamy, D. Bailey: University of Greenwich, School of Science, Central Avenue Chatham Maritime, Kent ME4 4TB

D. salina is meanwhile a potential source to produce glycerol: *D. salina* cells can accumulate up to 80% of their biomass as glycerol, depending on biological and environmental conditions [5]. This comes about as a characteristic response of *Dunaliella* to salinity stress, which is able to adjust the intracellular concentration of glycerol by regulating carbon flux between starch production in the chloroplast and the accumulation of glycerol in the cytoplasm. Glycerol is synthesised via both photosynthesis and starch degradation, and serves as an osmolyte responding to changes in environmental osmotic pressure [6-8]. Glycerol is currently used in food, feed, pharmaceutical and cosmetic industries and can also be used as a 'green chemical' feedstock to replace a variety chemicals currently derived from fossil fuels such as: ethylene and propylene glycols, 2,3-butanediol, epichlorohydrin and acrolein [9]. More recently glycerol has found applications as an emerging biofuel for an entirely new environmentally-sustainable, biofuel industry [10]. Although the gross calorific value or higher heating value (HHV) of glycerol is only 18 MJ kg^{-1} [11], it has a favourable volumetric energy density compared to other biofuels, and the McNeil combustion cycle allows it to be combusted for cogeneration at high efficiencies and with very low emissions without chemical alteration or the addition of combustion enhancers [10].

Commercial viability of an algal biorefinery needs to support the integration of large volume, low value microalgae biofuel production with high value co-products or by-products, without saturating any likely markets [12]. The product portfolio afforded by cultivating *Dunaliella* offers advantages for testing the commercial viability of an algal biorefinery: (a) there are multiple markets for glycerol either as a biofuel or chemical feedstock; this should allow production to suit different markets according to price; (b) it is possible to manipulate culture conditions to produce suites of different compounds flexibly from *Dunaliella* to meet market requirements, sustainably [4]. However, a major challenge for all algae biorefineries resides in cost effective harvesting [13, 14]. It has been suggested that 20 to 30% of the costs of microalgal biomass is due to the costs of harvesting [15-17], but estimates as high as 50% of microalgal biomass cost have been given [13].

Algal cells can be concentrated from culture broth using a variety of techniques that include gravity sedimentation, cross-flow filtration, coagulation, flocculation and centrifugation [18]. For many pilot and commercial scale processes one or more of these techniques has been proven effective. *Dunaliella* cells are approximately ~10 μm in diameter [5,19], and neutrally buoyant in a high specific gravity, high viscosity brine [5,18]. Also cell densities in large-scale cultures are usually < 1 g l^{-1} [20-22], therefore very large volumes need to be processed. But of more importance: unlike most of the green microalgae species, *Dunaliella* cells lack a rigid cell wall, instead possessing a thin elastic plasma membrane enriched in free sterols able to rapidly adapt to changes in external salt concentration [5,23].

Current harvesting technology for *Dunaliella* uses stacked disk clarifier centrifuges [16,24,25], which are the most common industrial centrifuges [26,27]. A disc-stack centrifuge consists of a relatively shallow cylindrical bowl containing a number (stack) of closely spaced metal cones (discs) that rotate with the bowl. The mixture to be separated is fed to the centre of the stack of discs and under the influence of centrifugal force the dense phase travels outwards on the underside of the discs and the lighter phase is displaced to the centre. Materials of different densities are separated into thin layers, and the narrow flow channel of 0.4-3 mm between the closely spaced discs means that the distance materials must travel for this separation to occur is small [26,28]. Disc-stack centrifuges are ideally suited for separating particles of the size (3 -30 μm) and concentration (0.02 - 0.05%) [20] typical of algal cells found in commercial growth systems [27,29]. However, they shear and lyse the *Dunaliella* cells during the separation cycle because of the combination of high g-forces (11,000 – 15,000 g) and hydrodynamic stresses [27,30-32]. For the harvesting of cell biomass enriched in β-carotene this has not been a problem: β-carotene accumulates as droplets in the periphery of chloroplasts and remains within the algal biomass during separation: it is readily recoverable. Indeed Ben-Amotz and Avron [25] found continuous-flow centrifugation with automatic discharge was one of the most effective methods for *Dunaliella* harvesting for biomass enriched in β-carotene. However, if the cells rupture, usually the case with clarifier centrifuges or filtration, glycerol and other water soluble compounds from cytosol would leak to the surrounding water, making it difficult to recover economically from the resulting dilute solution in the brine.

In this paper we present results from centrifugation experiments aimed at examining the effect of increasing g-force on the harvesting of *Dunaliella* cells using cytosolic glycerol as a marker for evidence of cell rupture. The work also used mechanistic calculations to examine the forces acting on a cell during centrifugation that may results in cell rupture.

2 Methods

2.1 Cell Culture

The green microalga *Dunaliella salina* (Dunal) Teodoresco CCAP 19/30 was obtained from the Culture Collection of Algae and Protozoa (CCAP, Scotland, UK) and maintained in a temperature controlled growth chamber at 23 ± 2 °C. Illumination was provided under a 12/12 h light/dark cycle by cool white fluorescent lamps with a light intensity of ~45 μmol s^{-1} m^{-2} photosynthetically active irradiation (PAR). *D. salina* was cultured in modified Johnson's medium [33,34] with 2.0 M NaCl. Cultures were maintained in stationary 250 ml shake flasks containing 100 ml medium for 10 days before harvesting with centrifugation.

2.2 Centrifugation

Aliquots (1.5 ml) of algal culture broth were centrifuged using a bench top micro-centrifuge (Eppendorf 5415R) at room temperature. Centrifugation was carried out at a representative selection of different speeds across range of the micro-centrifuge (1000, 2000, 3000, 5000, 7000, 9000, 11000, 13000, and 15000 g) for 10 min and each centrifugation condition was tested at least in triplicate. After centrifugation the supernatant was removed for analysis and the cell pellet was carefully re-suspended into fresh medium.

2.3 Cell Density

Cell densities of the culture broth, the new algal suspension and the centrifugation supernatant were measured by counting the intact cell number using a haemocytometer.

2.4 Glycerol Determination

The amounts of glycerol in the harvested cell pellet and centrifugation supernatant were both determined using the sodium periodate reagent method [35]: Cell pellets were re-suspended in 1.5 ml water and 0.2 ml chloroform followed by vigorous mixing. The water phase containing glycerol was recovered by centrifugation at 10,000 g for 10 min at room temperature. A series of glycerol standard solutions were prepared and 0.2 ml of each working standard and the glycerol sample were treated with 1 ml of periodate reagent (65 mg $NaIO_4$ in 90 ml of water, 10 ml acetic acid, and 7.7 g ammonium acetate) and 2.5 ml of acetylacetone reagent (2.5 ml of acetylacetone in 247.5 ml of isopropanol). All solutions were placed in a water bath at 60 °C for 30 minutes and stirred manually. The solutions were allowed to cool and the absorbance was measured using a UV/Vis spectrometer at wavelength of 410 nm. GC-MS analysis was used to confirm glycerol identity [36]: A 0.2 μl aliquot of the derivatised extract was injected into an Agilent DB5 capillary column (30 m × 0.25 mm × 0.25 μm). The inlet temperature was set at 220 °C. After a 5-minute solvent delay, initial GC oven temperature was set at 60 °C. Two minutes after injection, the GC oven temperature was raised to 240 °C (6 °C min^{-1}), and finally held at 250 °C for 6 min. Helium was used as the carrier gas with a constant flow rate set at 1 ml min^{-1}. The measurements were made with positive electron impact ionisation (70 eV) in the full scan mode (m/z 30-550). The detected peaks were identified by matching samples with spectra of the glycerol standard and using the GC-MS Metabolites Spectral Database (NIST 2005) mass spectral library.

3 Results, discussion and calculations

3.1 Experimental results

The total intact cells after centrifugation in both the supernatant liquid and settled pellet, together with that initially in culture media, for a range of g-forces in a bench-top centrifuge are shown in Figure 1. Across the whole g-force range tested, only a very small number of cells remained in the supernatant with the majority of the intact cells existing in the harvested cell pellets. Using an applied g-force of 3000 g, a cell paste ~60% solids was harvested comprising nearly 100% of the total cell biomass present mainly in the form of intact cells (7.75×10^6 cells, 6.01 ± 0.07 g l^{-1}). However, the number of intact cells recovered in the cell pellets decreased with increasing g-force, especially when the g-force was above 5000 g. When the g-force increased to 9000 g, the number of intact cells observed was only ~60% of that in the original culture broth. Therefore, although a higher g-force is usually used to harvest most microorganisms, for *D. salina*, a significant number of cells would be damaged during centrifugation at high g-forces (> 5000 g).

In Figure 2 the percentage of glycerol recovered in both the harvested cells and remaining in the supernatant together with the percentage of intact cells recovered are plotted against g-force. The amount of glycerol lost in un-harvested cells, was very low (0.03% to 1.3% of the

Figure 1 Effect of g-force on percentage glycerol and intact cells recovered.

Figure 2 Effect of g-force on percentage glycerol and intact cells recovered.

original glycerol present) compared with the amount of glycerol found in harvested cells or in the supernatant from ruptured cells, the concentration reducing with increasing g-force. Cell biomass comprising intact cells declined steadily and increasing quantities of glycerol were released to the culture medium with increasing g-force. When the g-force was no higher than 5000 g, the glycerol recovery rate was ~90% of the total. The maximum recovery of glycerol was obtained at ~2000 g in this study. With g-forces ≥ 9000 g, only ~60% of the glycerol was recovered, which was in line with the amount of intact cells harvested at such high g-forces.

The hydrodynamic stress caused by centrifugation with a bench-top centrifuge ruptured cells of *D. salina* at centrifugal forces ≥5000 g and ~40% of the cells were ruptured at centrifugal forces ≥ 9000 g. There was a good correlation between the amount of glycerol released to the medium and the number of cells ruptured, and only ~60% of the intracellular cytosolic marker glycerol was recovered at g forces ≥ 9000 g. This suggests the amount of glycerol increased in the supernatant was only caused by the increased number of ruptured cells whilst little glycerol would be released by the un-ruptured intact cells. Not all *Dunaliella* species may respond in the same way. Cells of *D. tertiolecta* for instance have been reported to have a specific transport protein for taking up exogenous glycerol [37] or excreting it [38]. Nevertheless Chow, *et al.* [38] recently described the continuous extraction

of glycerol by centrifugation using cells of *D. tertiolecta*. Applied centrifugal forces of 10000 g were used for 20 min in a bench-top centrifuge to harvest *D. tertiolecta* cells and most of the glycerol was found in the supernatant rather than in the harvested cell pellets. This would be as anticipated based on the results presented here. Adopting their approach for recovering cellular contents from the bulk medium, however, may require more energy in the long term than recovery from the concentrated cell pellet [14,18,27].

3.2 Calculation of hydrodynamic forces during centrifugation

The g-force to rupture *Dunaliella* cells in a laboratory centrifuge was found to be 5000 g in this study. Cells can experience a number of forces during centrifugation:

- Hydrodynamic stresses due turbulence
- Viscous drag
- The increase in cell 'weight' due to the 'centrifugal force'
- Hydrostatic pressure due to liquid head
- Pressure due to the 'weight' of cells in the pellet of recovered cell material

A range of calculations were carried out to examine the various hydrodynamic forces acting on the algal cells during centrifugation.

3.2.1 Estimate of cell membrane thickness

The cell membrane enclosing the *Dunaliella* cell has been described as being "almost invisible" [5]. Typically cell membranes are bimolecular layers with the thinnest being lipid bilayers (liposomes) at 4 -5 nm [39]. Aquaporins are channel proteins, which allow the diffusion of water and small solutes, such as glycerol, across biological membranes. Aquaporin molecules span the membrane to form pores [40,41]. The radius, r_p (nm), of an aquaporin can be calculated from the molecular mass in Daltons, M (Da) [42]:

Equation 1

$$r_p = 0.066\, M^{1/3}$$

The typical molecular mass of aquaporin is 28 kDa [40, 41]. If the membrane is assumed to be the thickness of aquaporin molecule a membrane thickness of 4 nm can be estimated in agreement with the typical thickness of a lipid bilayer.

3.2.2 Estimation of pressure to rupture cell membrane

The unicellular green alga *Chlamydomonas eugametos* which unlike *Dunaliella* has an algal cell wall surrounding the cell membrane was found to rupture at 95 atmospheres (9.63 MPa) [43]. The yield stress σ_y (Pa) for a thin walled sphere can be calculated using the following equation [39,44]

Equation 2

$$\sigma_y = \frac{pr_c}{2t}$$

Where p is the pressure exerted over the plane circular area (Pa), r_c is the radius of the cell (sphere) (m) and t is the thickness of the wall or membrane (m). For C*hlamydomonas eugametos* a microalgal wall stress of 6300 atmospheres (638 MPa) was calculated by Carpita [43] from equation 2 for a cell radius of 8 μm and a cell thickness of 60 nm. The bounding plasma membrane in cell wall-less *Dunaliella* is considerable thinner than that of the combined membrane and extracellular cell wall in C*hlamydomonas eugametos* and a lower pressure will rupture the cells. Assuming similar strengths of their boundary molecular architecture, albeit they have different molecular compositions, and rearranging equation 2 the pressure to rupture a *Dunaliella* cell membrane was calculated to be 1.0 MPa, for a cell radius of 5 μm, a cell thickness 4 nm and yield strength of 638 MPa.

3.2.3 Cell velocity and potential forces during centrifugation

In sedimentation gravitational forces cause liquid or solid particles to separate from a liquid of different density, but the process can be extremely slow especially if density difference or particle size is small. Sedimentation can be described by Stokes' Law which assumes that sedimentation velocity is proportional to the square of the (Stokes') radius of the cells and the difference in density between the micro-algal cells and the medium as shown below:

Equation 3

$$\text{Settling velocity} = \frac{2}{9}\, g_n\, \frac{r_c^2}{\mu}\, (\rho_s - \rho_l)$$

Where g_n is standard acceleration due to gravity (9.81 m s^{-2}) r_c is cell radius (m), μ is fluid dynamic viscosity (Pa s) and ρ_s and ρ_l are the solid and liquid densities (kg m^3). In centrifugation, gravity is replaced as the force driving separation by a much greater 'centrifugal force', thus greatly reducing separation time. The force

is normally expressed as g-force (g) the acceleration experienced by the body expressed as a multiple of the standard acceleration due to gravity, therefore for centrifugation g_n is replaced in equation 3 by g_c (ms^{-2}), the product of g and g_n

Stokes' law holds for spheroid shapes, but micro-algae are most often not spherical having a diverse range of shapes [45-47]. The shape of the *Dunaliella* cell was assumed to be spherical with a diameter of 10 μm. The density and viscosity of 1000 kg m^3 and 1000 μPa s [48] was assumed for the growth medium. The cytoplasm of marine micro-algae has a density between 1030 and 1100 kg m^{-3} [46], the density of cyanobacteria is between 1082 and 1104 kg m^{-3} [49], marine diatom and dinoflagellates between 1030 and 1230 kg m^{-3} and the freshwater green micro-algae *(Chlorococcum)* between 1040 and 1140 kg m^{-3} [50]. The density of micro-algae is thus close to that of water and of salt water (998 and 1024 kg m^{-3} at 20° C respectively) [48,51,52] and therefore there is little density difference driving micro-algal settlement. The density of the *Dunaliella* was assumed to be 1260 kg m^3 the density of glycerol [53] a major component of cells of *Dunaliella*. The velocity of *Dunaliella* cell was calculated using Equation 3 at 0.04 m s^{-1} for 3000 g and 0.13 m s^{-1} for 9000 g.

3.2.4 Hydrodynamic stresses from turbulence

Reynolds number (R_e) has been described as one of the most basic concepts in fluids and the most important dimensionless number [54]. It is the ratio of inertia forces to viscous forces within the fluid. At low Reynolds numbers viscous forces dominate and flow is laminar, characterised by smooth motion of the fluid in layers, without significant mixing between layers. Turbulent flow occurs when inertial forces dominate, producing circulating currents or eddies, and there is exchange of momentum across the primary direction of fluid flow [55]. Reynolds number and can be calculated by [55]:

Equation 4

$$R_e = \frac{\rho_l v d_c}{\mu}$$

where v is the velocity of the fluids relative to the cell (ms^{-1}), d_c is cell diameter (m), ρ_l liquid density (kgs^{-1}) and μ viscosity (Pa s). For a *Dunaliella* cell of 10 μm diameter in a 9000 g centrifuge the Reynolds number is 1.3 , and therefore the flow is laminar and the cell will not experience turbulent hydrodynamic forces, such as micro-eddies, that could damage the cell membrane [18,27].

3.2.5 Viscous Drag

The viscous drag F_d (N) on a cell can be expressed as [39]:

Equation 5

$$F_d = 6\pi\mu r_c$$

The viscous drag is therefore 4.0×10^{-09} N at 3000 g and 1.0×10^{-08} N at 9000 g equivalent to pressure acting on the cell of 51 Pa and 153 Pa considerable below the calculated pressure to fracture the cell of 1.0 MPa. The force exerted by viscous drag is also below the force estimated by Lee *et al.* (2012) as the minimum to force to cause noticeable leakage of cell content from microalgae of 11 μN.

3.2.6 The increase in cell weight due to the centrifugal force

The forces acting on a cell resting on surface due to its own mass increase with increasing 'centrifugal force'. The pressure acting on the cell due to its own mass and the 'centrifugal force' is 247 Pa at 3000 g and 742 Pa at 9000 again both considerably below the estimated pressure to rupture a cell of 1.0 MPa.

3.2.7 Hydrostatic pressure due to liquid head

The hydrostatic pressure (p_h) exerted on cell at the bottom of a tube in a centrifuge can be considerable and can be calculated by [55]:

Equation 6

$$p_h = h \rho g_c$$

where h (m) is the height of the liquid in the centrifuge tube. The height of the liquid in the experimental work was 30mm which at 3000 g is a hydrostatic pressure of 0.9 MPa, less than the estimated rupture pressure for *Dunaliella*. At 6000 g the hydrostatic pressure is 1.8 MPa greater than the estimated rupture pressure, and the hydrostatic pressure at 9000 g is 2.7 MPa over twice the cell rupture pressure.

3.2.8 Pressure due to the weight of cells in the pellet

The pressure of the cells in the pellet acting on the cells at the bottom of the pellet increases with the height of the pellet, the 'centrifugal force' and the density of the cell (kg m^{-3}). Microalgal material has a relative low density,

in this case 1260 kg m^{-3}, and a pellet height of 11.6 mm is required at 9000 g to achieve the pressure equivalent to a rupture pressure of 1.0 MPa, considerable greater than the height of the pellets recovered in laboratory centrifuge experiments (< 5 mm). The force due to pellet pressure for a 5 mm pellet was calculated 185 Pa and 554 Pa for 3000 and 9000 g respectively

3.2.9 Discussion on calculation of hydrodynamic forces

The main forces exerted on algal cells at 3000 g or 9000 g in centrifuge obtained from the above calculations are summarised in Table 1.

Table 1: Calculated forces exerted on algal cells in a bench-top centrifuge.

Centrifugal g-force	Viscous drag	Increased cell 'weight'	Liquid head	Pellet pressure
3000 g	51 Pa	247 Pa	0.9 MPa	185 Pa
9000 g	153 Pa	742 Pa	2.7 MPa	554 Pa

The calculations indicate that cell disruption at 9000 g was primarily due to the hydrostatic head of liquid above the recovered cells with the other forces such as viscous drag being considerable smaller. The calculated centrifugal shear stress to rupture *Dunaliella* cells is in agreement with laboratory experiments. The calculation predicts little rupture of cells at 3000 g, but rupture of the *Dunaliella* cell membrane at 6000 g and 9000 g with consequential loss of glycerol to the medium. The laboratory centrifuge with a known depth of liquid could be the basis of a method to calculate the rupture pressure of a range of organic cells.

4 Conclusion

Disruption of *Dunaliella salina* cells occurred due to high hydrostatic pressure in the centrifugation process. A decrease in the number of intact cells was observed when the centrifugal g-force increased above 5000 using a bench-top centrifuge. The yield of glycerol recovered in intact cells also proved to be affected significantly by centrifugation conditions. With centrifugal g-force higher than 9000, there was ~ 40% loss of the intracellular glycerol to the medium compared with g-forces below 5000. Therefore, for recovery of glycerol in intact cells, it is critical to optimise the centrifugation conditions in the harvesting of *Dunaliella salina*.

Acknowledgement: This work was carried out with support from Ecotec 21, Kebbi State Nigeria and University of Greenwich.

Conflict of interest: The authors have nothing to disclose.

References

[1] Milledge J.J., Commercial application of microalgae other than as biofuels: A brief review, Rev. Environ. Sci. Biotechnol., 2011, 10, 31-41

[2] Vanthoor-Koopmans M., Wijffels R.H., Barbosa M.J., Eppink M.H.M., Biorefinery of microalgae for food and fuel, Biores. Technol., 2013, 135, 142-149

[3] Milledge J.J., Heaven S., Methods of energy extraction from microalgal biomass: A review, Rev. Environ. Sci. Biotechnol., 2014, 13, 301-320

[4] Harvey P., Abubakar A.L., Xu Y., Bailey D., Milledge J.J., Swamy R., *et al.*, The CO_2 microalgae biorefinery: High value products from low value wastes using halophylic microalgae in the d-factory. Part1: Tackling cell harvesting in European Biomass Conference, Hamburg, 2014

[5] Ben-Amotz A., Polle J.E.W., Subba Rao D.V., The alga Dunaliella: Biodiversity, physiology, genomics and biotechnology, Science Publishers, Enfleld NJ, 2009

[6] Cowan A.K., Rose P.D., Horne L.G., Dunaliella-salina - a model system for studying the response of plant-cells to stress, J. Exp. Bot., 1992, 43, 1535-1547

[7] Goyal A., Osmoregulation in Dunaliella, part i: Effects of osmotic stress on photosynthesis, dark respiration and glycerol metabolism in Dunaliella tertiolecta and its salt-sensitive mutant (hl 25/8), Plant Physiol. Biochem., 2007, 45, 696-704

[8] Goyal A., Osmoregulation in Dunaliella, part ii: Photosynthesis and starch contribute carbon for glycerol synthesis during a salt stress in Dunaliella tertiolecta, Plant Physiol. Biochem., 2007, 45, 705-710

[9] Tan H.W., Abdul Aziz A.R., Aroua M.K., Glycerol production and its applications as a raw material: A review, Renew. Sustain. Energy Rev., 2013, 27, 118-127

[10] Harvey P.J., Psycha M., Kokossis A., Abubakar A.L., Trivedi V., Swamy R., *et al.*, Glycerol production by halophytic microalgae: Strategy for producing industrial quantities in saline water in 20th European Biomass Conference and Exhibition, 85 - 90, 2012

[11] Domalski E.S., Selected values of heat of combustion and heat of formation of organic compounds containing the elements C, H, N, O, P and S, J. Phy. Chem. Ref. Data, 1972, 1, 221-277

[12] Baghel R.S., Trivedi N., Gupta V., Neori A., Chennur R.R., Lali A.M., *et al.*, Biorefining of marine macroalgal biomass for production of biofuel and commodity chemicals, Green Chem., 2015,

[13] Greenwell H.C., Laurens L.M.L., Shields R.J., Lovitt R.W., Flynn K.J., Placing microalgae on the biofuels priority list: A review of the technological challenges, J. R. Soc. Interface, 2010, 7, 703-726

[14] Coons J.E., Kalb D.M., Dale T., Marrone B.L., Getting to low-cost algal biofuels: A monograph on conventional and cutting-edge harvesting and extraction technologies, Algal Res., 2014, 6, Part B, 250-270

[15] Mata T.M., Martins A.A., Caetano N.S., Microalgae for biodiesel production and other applications: A review, Renew. Sust. Energ. Rev., 2010, 14, 217-232
[16] Molina Grima E., Belarbi E.-H., Acien-Fernandez F.G., Robles-Medina A., Yusuf C., Recovery of microalgal biomass and metabolites: Process options and economics, Biotechnol. Adv., 2003, 20, 491-515
[17] Verma N.M., Mehrotra S., Shukla A., Mishra B.N., Prospective of biodiesel production utilizing microalgae as the cell factories: A comprehensive discussion, Afr. J. Biotechnol., 2010, 9, 1402-1411
[18] Milledge J.J., Heaven S., A review of the harvesting of micro-algae for biofuel production, Rev. Environ. Sci. Biotech., 2013, 12, 165-178
[19] Chengala A.A., Hondzo M., Troolin D., Lefebvre P.A., Kinetic responses of Dunaliella in moving fluids, Biotech. Bioeng., 2010, 107, 65-75
[20] Zamalloa C., Vulsteke E., Albrecht J., Verstraete W., The techno-economic potential of renewable energy through the anaerobic digestion of microalgae, Biores. Technol., 2011, 102, 1149-1158
[21] Zhu L.D., Hiltunen E., Antila E., Zhong J.J., Yuan Z.H., Wang Z.M., Microalgal biofuels: Flexible bioenergies for sustainable development, Renew. Sust. Energ. Rev., 2014, 30, 1035-1046
[22] Borowitzka M.A., Culturing of microalgae in outdoor ponds, in Anderse R.A. (Ed.), Algal culturing techniques, Elsevier, London, 2005
[23] Peeler T.C., Stephenson M.B., Einspahr K.J., Thompson G.A., Lipid characterization of an enriched plasma-membrane fraction of Dunaliella salina grown in media of varying salinity, Plant Physiol., 1989, 89, 970-976
[24] Ben-Amotz A., Bio-fuel and CO_2 capture by algae, NASA, 2008
[25] Ben-Amotz A., Avron M., The biotechnology of mass culturing of Dunaliella for products of commercial interest, eds Cresswell R.C., Ress T.A.V., Shah N., Longman Scientifc and Technical Press, London, 90-114, 1989
[26] Perry R.H., Chilton C.H., Chemical engineers' handbook, McGraw Hill, Tokyo, Fifth Ed, 1973
[27] Milledge J.J., Heaven S., Disc stack centrifugation separation and cell disruption of microalgae: A technical note, Environ. Nat. Resour. Res., 2011, 1, 17-24
[28] Mannweiler K., Hoare M., The scale-down of an industrial disk stack centrifuge, Bioprocess Eng., 1992, 8, 19-25
[29] Axelsson H., Recent trends in disc bowl centrifuge development, Filtr. Sep., 2000, 37, 20-23
[30] Silva H.J., Cortifas T., Ertola R.J., Effect of hydrodynamic stress on Dunaliella growth, J. Chem. Technol. Biotechnol., 1987, 40, 41-49
[31] Boychyn M., Yim S.S.S., Bulmer M., More J., Bracewell D.G., Hoare M., Performance prediction of industrial centrifuges using scale-down models, Bioprocess Biosyst. Eng., 2004, 26, 385-391
[32] Garcia Camacho F., Gallardo Rodriguez J.J., Sanchez Miron A., Belarbi E.H., Chisti Y., Molina Grima E., Photobioreactor scale-up for a shear-sensitive dinoflagellate microalga, Process Biochem., 2011, 46, 936-944
[33] Johnson M.K., Johnson E.J., MacElroy R.D., Speer H.L., Bruff B.S., Effects of salts on the halophilic alga Dunaliella viridis, J. Bacteriol., 1968, 95, 1461-1468
[34] Borowitzka M.A., Algal growth media and sources of cultures, Microalgal biotechnology, eds Borowitzka L.J., Borowitzka M.A., Cambridge University Press, Cambridge, 1988
[35] Chen H., Lao Y.-M., Jiang J.-G., Effects of salinities on the gene expression of a (nad+)-dependent glycerol-3-phosphate dehydrogenase in Dunaliella salina, Sci. Total Environ., 2011, 409, 1291-1297
[36] Lamers P.P., Metabolomics of carotenoids accumulation in Dunaliella salina, PhD, Wageningen University, 2011
[37] Lin H.X., Fang L., Low C.S., Chow Y., Lee Y.K., Occurrence of glycerol uptake in Dunaliella tertiolecta under hyperosmotic stress, FEBS J., 2013, 280, 1064-1072
[38] Chow Y.Y., Goh S.J., Su Z., Ng D.H., Lim C.Y., Lim N.Y., *et al.*, Continual production of glycerol from carbon dioxide by Dunaliella tertiolecta, Bioresour. Technol., 2013, 136, 550-555
[39] Boal D.H., Mechanics of the cell, Cambridge University Press, Cambridge ; New York, 2nd Ed, 2012
[40] Preston G.M., Carroll T.P., Guggino W.B., Agre P., Appearance of water channels in xenopus oocytes expressing red cell chip28 protein, Science, 1992, 256, 385-387
[41] Oliva R., Calamita G., Thornton J.M., Pellegrini-Calace M., Electrostatics of aquaporin and aquaglyceroporin channels correlates with their transport selectivity, Proc. Nat. Acad. Sci., 2010, 107, 4135-4140
[42] Erickson H.P., Size and shape of protein molecules at the nanometer level determined by sedimentation, gel filtration, and electron microscopy, Biological Procedures Online, 2009, 11, 32-51
[43] Carpita N.C., Tensile-strength of cell-walls of living cells, Plant Physiol., 1985, 79, 485-488
[44] Hibbeler R.C., Fan S.C., Mechanics of materials, Prentice Hall, Singapore, 8th ed. in SI units Ed, 2012
[45] Peperzak L., Colijn F., Koeman R., Gieskes W.W.C., Joordens J.C.A., Phytoplankton sinking rates in the rhine region of freshwater influence, J. Plankton Res., 2003, 25, 365-383
[46] Smayda T.J., The suspension and sinking of phytoplankton in the sea, in Barnes H. (Ed.), Oceanography and marine biology annual review, George Allen & Unwin, London, Vol 8, 353-414, 1970
[47] Sournia A. ed, Phytoplankton manual, UNESCO, Paris, 1978
[48] Kestin J., Sokolov M., Wakeham W.A., Viscosity of liquid water in the range -8 to 150 °C, J. Phys. Chem. Ref. Data, 1978, 7, 941-948
[49] Kromkamp J., Walsby A.E., A computer-model of buoyancy and vertical migration in cyanobacteria, J. Plankton Res., 1990, 12, 161-183
[50] Van Lerland E.T., Peperzak L., Separation of marine seston and density determination of marine diatoms by density gradient centrifugation, J. Plankton Res., 1984, 6, 29-44
[51] Millero F.J., Lepple F.K., The density and expansibility of artificial seawater solutions from 0 to 40°C and 0 to 21 chlorinity, Marine Chem., 1973, 1, 89-104
[52] El-Dessouky H.T., Ettouney H.M., Fundamentals of salt water desalination Elsevier, Amsterdam, 2002
[53] Harrison R.D., Book of data, Nuffield Advanced Science, 1972
[54] Lowe S.A., Omission of critical Reynolds number for open channel flows in many textbooks, J. Professional Iss. Eng. Edu. Practice, 2003, 129, 58-59
[55] Coulson J.M., Richardson J.F., Coulson & Richardson's chemical engineering . Fluid flow, heat transfer and mass transfer, Elsevier, Oxford, 6 Ed, 1999