

Microalgae Cultivation for Lipids and Carbohydrates Production

Andrea Visca^a, Fabrizio Di Caprio^{*a}, Roberta Spinelli^a, Pietro Altimari^a, Agnese Cicci^b, Gaetano Iaquaniello^b, Luigi Toro^a, Francesca Pagnanelli^a

^a Dipartimento di Chimica, Università "Sapienza" di Roma, Piazzale Aldo Moro 5, 00185, Roma, Italia.

^b Bio-P srl, Via di Vannina 88, Rome, Italy
fabrizio.dicaprio@uniroma1.it

Microalgae are photoautotrophic microorganisms that can produce energy both by using sunlight, water and CO₂ (phototrophic metabolism) and by using organic sources such as glucose (heterotrophic metabolism).

Heterotrophic growth is a key factor in microalgae research, due to its increased productivity and the lower capital and operative costs compared to photoautotrophic growth in photobioreactors.

Carbohydrate production from microalgae is usually investigated for the production of biofuels (e.g. bioethanol) by successive fermentation, but also other applications can be envisaged in biopolymers.

In this work an increment in carbohydrate purity after lipid extraction was found. Protein hydrolysis for different microalgae strains (*Scenedesmus* sp. and *Chlorella* sp.) was investigated. Microalgae were cultivated under photoautotrophic or heterotrophic conditions, collecting biomass at the end of the growth. Biomass samples were dried or freeze dried and used for carbohydrate and lipid extraction tests. Lipid extraction was achieved using different organic solvents (methanol-chloroform and hexane-2propanol). Basic protein hydrolysis has been carried out testing different temperatures and NaOH concentrations values. Lipids were spectrophotometrically quantified, while residual biomass was saccharificated and the total amount of sugars was measured.

Significant differences about the purity of extracted carbohydrates were found comparing dried with freeze dried biomass. However, not a very promising purification of carbohydrates was achieved after protein hydrolysis, asking for further analysis.

1. Introduction

CO₂ emissions and environmental pollution associated to the use of fossil fuels are widely recognised as a threat to the global health of the planet (Yen et al., 2013). Even if the CO₂ produced by natural and human activity can be converted to new biomass by plants, it is not enough to balance the overall anthropogenic emission. This is why increasing attention has been given to microalgae, due to their faster CO₂ fixation compared to plants (Ho et al., 2011). Moreover, microalgae produce different valuable components, such as carbohydrates, long chain fatty acids, pigments and proteins (Yen et al., 2013). Carbohydrate fraction consists mostly of cellulose and starch without any lignin residue, so it can be easily used for the fermentation process (John et al., 2011).

One of the problems slowing the commercialization and diffusion of microalgal cultivation is the high cost of microalgae culture systems (photobioreactors) ensuring the light needs of cultures typically living in autotrophic conditions (Altimari et al., 2013). This problem can be avoided by growing microalgae in heterotrophic way. Heterotrophic metabolism takes place when an organic carbon source is provided. In this case, microalgae start growing using glycolysis pathway (Richmond, 2004). For example, *Chlorella* cells have an inducible active transport system for glucose, which is positively activated by glucose (Tanner, 1969; Haass & Tanner, 1974; Fenzl et al., 1977).

Heterotrophic conditions could however result in lower maximum specific growth rate in comparison with phototrophic growth (Ogawa & Aiba, 1981; Kobayashi et al., 1992; Di Caprio et al., 2016). This could be

partially explained by substrate inhibition (Richmond, 2004) requiring specific optimization of glucose/nitrate ratio (Altimari et al., 2014; Pagnanelli et al., 2014).

Heterotrophic growth of some microalgae has been used for efficient production of biomass and some metabolites such as lipids (Miao and Wu, 2006) and carbohydrates (Di Caprio et al. 2015a). Lipids can be used as a source for biofuels, as building blocks in chemical industry, and edible oils for the food and health market. Application of biorefinery concept (exploiting all products contained in biomass) can further enhance microalgal cultivation feasibility (Di Caprio et al., 2015b). Downstream operations should be mild, inexpensive and low energy consuming, avoiding product damages and enforcing process economy (Vanthoor-Koopmans et al., 2012)

Solvent extraction is one of the most commonly employed methods in lipid extraction for biodiesel production. (Singh, J.; Gu, S., 2010; Halim et al., 2012; Grima et al., 2013). However, solvent extraction presents problems related to energy consumption, environmental pollution, and safety risks. Moreover, in order to increase the extraction efficiency, often solvent heating is required, making it even more energy intensive (Medina et al., 1998), and often requires the presence of a polar solvent, such as methanol or 2-propanol, as they can disrupt the higher energy complex between neutral and polar lipids. (Halim et al., 2012; Medina et al., 1998; Bligh et al., 1959) This leads to another issue: oils recovered in this way are rich in other more polar biomolecules, such as chlorophyll, free fatty acids, phospholipids, sterols and gangliosides, which normally interfere with the final biodiesel product (Grima et al., 2013).

Besides lipids, microalgal biomass can be a source for proteins, which can be used in food, feed, health and bulk chemical market, and carbohydrates for producing ethanol and other chemicals (Radakovits et al., 2010) Nevertheless, the success of these new bio-based productions will heavily depend on engineering solutions, developing innovative separation operations as crucial point of the whole process. Alkaline protein extraction from biomass is an important first step when studying the route from biomass, via protein and amino acids, to bulk chemicals. After protein hydrolysis, moreover, protease can be used to hydrolyse protein and peptides to single amino acids: this is an important step in biorefinery process, but still little explored (Sari et al., 2015).

The aim of this study was to reach the isolation and purification of carbohydrates from microalgae after a sequential extraction of lipids and proteins. Assuming the composition of the microalgal cell as lipids, proteins and carbohydrates, the extraction of lipids and proteins should allow to obtain purified carbohydrates from microalgae.

2. Materials and Methods

2.1 Strains and cultivation conditions

The *Scenedesmus* sp. strain was selected in Siracusa (Sicily, Italy) and maintained in Petri dish in MBG11 (modified BG11 medium, with a reduced NaNO_3 concentration of 0.3 g/L) solid medium (Di Caprio et al. 2015). The *Chlorella* sp. strain was selected in Rome (Lazio, Italy) and maintained in the same way. Microalgae were firstly transferred from the Petri dish to 500 mL flasks in MBG11 liquid medium and then inoculated in 4000 mL column reactors using 1:10 dilution ratio. Microalgae were cultivated in photoautotrophic conditions, under constant illumination (24 h) with $80 \pm 10 \mu\text{E m}^{-2} \text{s}^{-1}$ and feed with 0.5 L/min of CO_2/air (0.05/1 v/v). Heterotrophic condition has been obtained transferring a phototrophic microalgal culture (on exponential growth phase) to a MBG11 medium added with 10 g/L of glucose as organic carbon source, and with no illumination provided.

Microalgae concentration was measured by filtration of 10 mL of sample on 0.45 μm acetate cellulose filter. The filters were dried at 105 °C and then weighted, and the microalgal concentration reported as g/L of biomass concentration. Algal culture was harvested by sedimentation and centrifugation at 3000 rpm for 10-15 minutes. Cell pellets were re-suspended in deionized water and washed three times via centrifugation and re-suspension to remove residual salts. The washed cells were dried at 105°C for 12 h or freeze dried and stored until further use.

2.2 Extraction and analytical determination of total lipid content

Lipid extraction was carried out in a glass tube (at room temperature), using hexane/2-propanol (H2P; 3:2 v/v) or trichloromethane/methanol solution (Folch; 9:4 v/v), for about 12-16 hours, using 200 mg of dried microalgae under magnetic stirring. At the end of the process the total extractable lipids were measured colourimetrically, and the residual biomass dried at 105°C for 12 h.

The determination of total lipid was achieved through sulpho-phospho-vanillin (SPV) colorimetric method (Byreddy et al., 2016). 1 mL of the total extracted lipids was transferred to a vessel, and the solvent evaporated. After this, 5 mL of a solution containing sulfuric acid (85% w/w) and vanillin 6 g/L were added, and the samples incubated at 37°C for 15 minutes. Then the absorbance was acquired at 530 nm wavelength by the UV-Visible spectrophotometer (Varian Cary 50 Scan).

2.3 Carbohydrate recovery

Samples of 100 mg of dried microalgae were put in a Pyrex tube with 1 mL of concentrated sulfuric acid (72% w/w) at 30 °C for 1 hour. Afterwards the sample was put in a glass vial with 28 mL of distilled water (in order to obtain a final concentration of 4%) and kept for 1 hour at 120 °C in autoclave. The sample was then quickly cooled and 1 mL was centrifuged at 8000 rpm for 5 minutes. The sugar concentration in the supernatant was then analyzed by Dubois method (Dubois et al. 1951).

2.4 Protein hydrolysis

The hydrolysis of proteins was conducted putting 100 mg of microalgae in 1 mL of NaOH at various concentrations (1 and 5 N) and various temperatures (20 and 100°C) for 1 h. At the end of the process, the sample was centrifuged at 3000 rpm for 5 min. The residual biomass was then washed 4-5 times with deionized water and dried at 105°C for 12 h.

3. Results and discussion

3.1 Biomass production

Microalgal strains were cultivated under both phototrophic and heterotrophic conditions, in order to compare the two different metabolisms (Figures 1). Both *Scenedesmus* sp. and *Chlorella* sp. strains show similar final concentration of biomass in phototrophic and heterotrophic conditions (about 1 g/L reached after 8 days). Specific growth rates (estimated by semi-logarithmic diagrams using only data following a linear relation) showed a decrease in heterotrophic conditions adopted (Table 1). The advantage of heterotrophic growth is not in increased grow rate, but it is in the increased biomass productivity. However, this difference can be observed (in this case) only in the last days of the cultivation: both phototrophic and heterotrophic reached about 0.75-1 g/L of algae concentration after 8-10 days, but both *Scenedesmus* sp. and *Chlorella* sp. phototrophic conditions are reaching a plateau due to growth inhibition caused by the turning of light into a limiting factor. On the other hand, in heterotrophic conditions the only limiting factor is the organic carbon source, that can be controlled by the operator, allowing to virtually reach infinite concentrations of algal biomass.

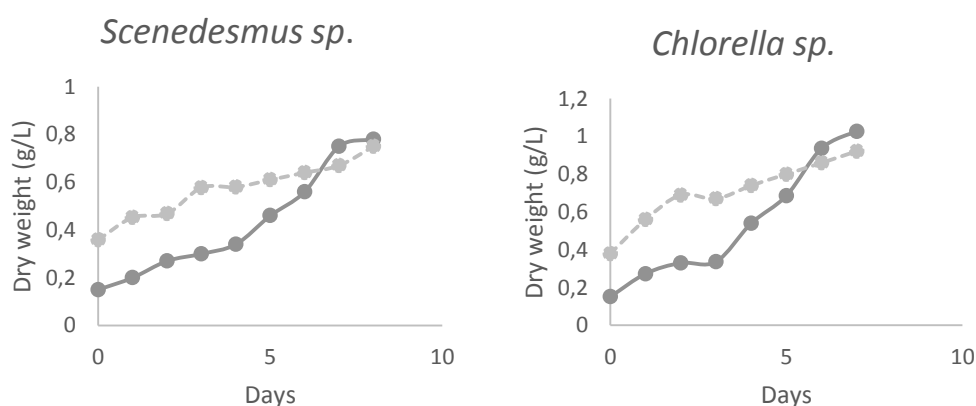


Figure 1. Biomass concentration (g/L) of *Scenedesmus* sp. and *Chlorella* sp. during time. Dark grey lines refer to phototrophic growth, while light grey lines refer to heterotrophic grow.

Table 1: μ (d^{-1}) of *Scenedesmus* sp. and *Chlorella* sp. under phototrophic and heterotrophic conditions

	Phototrophic condition	Heterotrophic condition
<i>Scenedesmus</i> sp.	0.219 (± 0.005)	0.100 (± 0.006)
<i>Chlorella</i> sp.	0.32 (± 0.05)	0.23 (± 0.04)

Further tests are now in course to optimise the organic carbon/N ratio in order to improve biomass growth in heterotrophic conditions.

3.2 Lipids extraction

In this work, the main focus on the lipids extraction was trying to increase carbohydrate purity by the elimination of one of the three macromolecules composing the microalgae: at the end of the process, the residual biomass should be composed only by carbohydrates and proteins.

Different strategies were used in order to achieve maximum carbohydrate recovery (expressed as residual solid on initial biomass weight) and purity (expressed as percentage of carbohydrates on residual solid).

Table 2: percentage of lipid extracted quantified through SPV method. Each column refers to a different microalgal strain, while each row refers to a different treatment: 1) dried biomass treated with CHCl₃/MeOH; 2) dried biomass treated with hexane/2-propanol; 3) freeze-dried biomass treated with CHCl₃/MeOH; 4) freeze-dried biomass treated with hexane/2-propanol;

	<i>Scenedesmus</i> sp.	<i>Chlorella</i> sp.
1) CHCl ₃ /MeOH	9.6% (±0.3)	7.5% (±0.3)
2) H2P	6.1% (±0.5)	5.6% (±0.9)
3) Freeze dry + CHCl ₃ /MeOH	6.9% (±0.3)	6.2% (±0.5)
4) Freeze dry + H2P	3.7% (±0.3)	5.6% (±0.3)

Table 2 summarizes the percentage of extracted lipids after each treatment. It is worth to observe how the chloroform/methanol solution gives higher efficiency on extracted lipids, compared to hexane/2-propanol, especially on dried biomass.

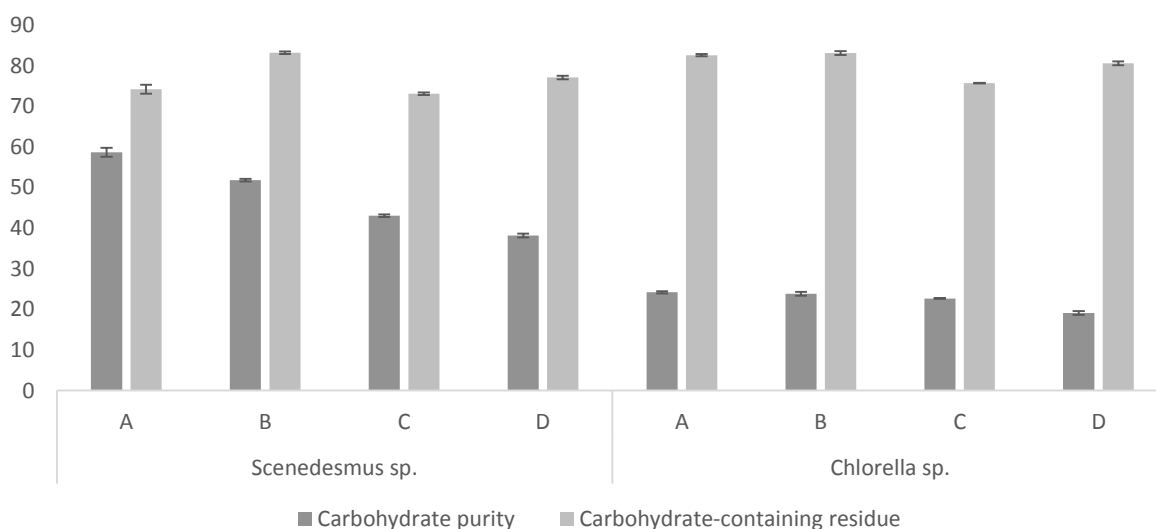


Figure 2: Percentage of carbohydrate purity (grey) and carbohydrate-containing residue (light grey). Each column refers to a different treatment: A) freeze-dried biomass treated with CHCl₃/MeOH; B) freeze-dried biomass treated with hexane/2-propanol; C) dried biomass treated with CHCl₃/MeOH; D) dried biomass treated with hexane/2-propanol.

In all extraction tests the *Scenedesmus* sp. biomass shows a better efficiency on carbohydrate extraction, probably due to its starting carbohydrates content, richest compared to the *Chlorella* sp. (30.5% carbohydrates on *Scenedesmus* sp., 17.7% on *Chlorella* sp.).

Combined action of freeze drying and solvent extraction seems to boost significantly the process for *Scenedesmus* sp. biomass, reaching a maximum in carbohydrate purity of 58.7 % in the freeze drying pre-treatment and chloroform/methanol solution. Even if the use of hexane/2-propanol doesn't allow achievement of the same value, it is still pretty close (51.8%), which, compared with the more healthy nature of the solvents, is a promising result. However, on *Chlorella* sp. biomass, freeze-drying process do not seem very useful: this is probably due to the different composition on cell wall. *Chlorella* sp. has probably a weaker cell wall compared to *Scenedesmus* sp., and this could justify the positive action of freeze-drying observed on the latter. Although these results are in contrast with other works (Guldhe et. al., 2014), where it is reported no

difference in lipid extraction yield comparing oven drying, freeze drying or sun drying for *Scenedesmus sp.* biomass, in our tests freeze dried biomass appeared more porous and friable than oven dried counterpart. A similar results has been obtained by Zhang and co-workers for starch drying (Zhang et al., 2014). This difference probably enhance solvent penetration in the stronger cell walls of *Scenedesmus sp.* biomass.

3.3 Protein hydrolysis

The residual biomass obtained after lipid extraction has been used for the sequential basic protein hydrolysis. The only biomass used for this test was *Scenedesmus sp.* dried one, after a lipid extraction with $\text{CHCl}_3/\text{MeOH}$.

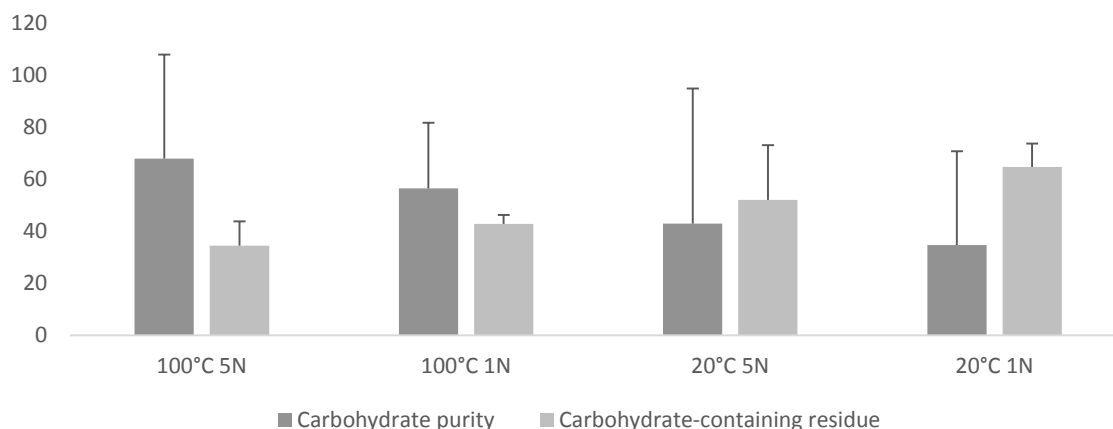


Figure 3: Percentage of carbohydrate purity (grey) and carbohydrate-containing residue (light grey) after protein hydrolysis. Each column refers to a different temperature and NaOH concentration.

Different possibilities have been screened, searching for the most effective combination of NaOH concentration and temperature for the protein hydrolysis, investigated with a factorial analysis. However the examined conditions have shown poor efficiency on carbohydrates purity increase, without a significant difference determined by ANOVA analysis ($\alpha=0.05$). This is due to the low reproducibility of the tests, as showed by the high error bars in Figure 3.

4. Conclusions

Different microalgal growth conditions have been compared. Growth rate and maximum biomass concentration have been obtained for phototrophic and heterotrophic cultivation for two different microalgal strains: *Chlorella sp.* and *Scenedesmus sp.* Both showed a similar biomass concentration in the two conditions, while the growth rate was higher in phototrophic cultivation.

Sequential separation of lipids and proteins showed promising results. By removing lipids, carbohydrate content was increased until 58.6 %. Efficiency of used biomass pre-treatments before lipid extraction depends by the type of used biomass. For *Scenedesmus sp.* freeze drying is advisable, while for *Chlorella sp.* a more cheaper common drying process can be used without relevant difference. For both strains, also if better results were obtained with chloroform/methanol, hexane/2-propanol gave comparable results in carbohydrate increment, as a consequence it is the recommended solvent mixture for its minor environmental impact.

Alkaline protein hydrolysis showed contrasting results: even if there is a little increase on carbohydrate purity, the high amount of error requires further tests to confirm these data.

Further tests will be required to improve protein hydrolysis efficiency, and treatment should be investigated also for *Chlorella sp.* biomass. Effect of different solvents usage for lipid extraction could affect protein hydrolysis efficiency, this is another factor which should be investigated in further tests, since in this work only *Scenedesmus sp.* biomass treated with chloroform/methanol has been tested for the successive protein hydrolysis.

References

Altimari, P., Pagnanelli, F., Toro, L., 2013. Application of structured population balance model for the numerical simulation of a continuous photobioreactor. *Chemical Engineering Transactions* 32, 1027-1032

- Altimari, P., Di Caprio, F., Toro, L., Capriotti, A.L., Pagnanelli, F. 2014. Hydrogen photo-production by mixotrophic cultivation of *Chlamydomonas reinhardtii*: Interaction between organic carbon and nitrogen. *Chemical Engineering Transactions* 38, 199-204
- Bligh, E. G.; Dyer, W. J., 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.*, 37, 911-917.
- Byreddy A. R., Gupta A., Barrow C.J., Puri M., 2016. A quick colorimetric method for total lipid quantification in microalgae. *J. Microbiol. Methods* 125, 28-32.
- Di Caprio F., Altimari P., Pagnanelli F., 2015a. Integrated biomass production and biodegradation of olive mill wastewater by cultivation of *Scenedesmus* sp. *Algal Res.* 9, 306-311
- Di Caprio F., Altimari P., Toro L., Pagnanelli F., 2015b. Effect of lipids and carbohydrates extraction on astaxanthin stability in *Scenedesmus* sp. *Chemical Engineering Transactions*, 43, 205-210.
- Di Caprio F., Visca A., Altimari P., Toro L., Masciocchi B., Iaquaniello G., Pagnanelli F., 2016. Two-stage process of microalgae cultivation for starch and carotenoid production. *Chemical Engineering Transactions* 49, 415-420
- Dubois M., Gilles K. A., Hamilton J. K., Rebers P. A., Smith F., 1956. Colorimetric Method for Determination of Sugars and Related Substances. *Anal. Chem.*, 28, 350-356
- Fenzl, F., Decker, M., Haass, D. & Tanner, W., 1977. Characterisation and partial purification of an inducible protein related to hexose proton co-transport of *Chlorella vulgaris*. *Eur. J. Biochem.*, 72, 509-14.
- Grima, E. M.; González, M. J. I.; Giménez, A. G., 2013. Solvent extraction for microalgae lipids. In *Algae for Biofuels and Energy*; Borowitzka, M. A., Moheimani, N. R., Eds.; Springer Netherlands: Dordrecht, 187-205.
- Guldhe A., Singh B., Rawat I., Ramluckan K., Bux F.. 2014. Efficacy of drying and cell disruption techniques on lipid recovery from microalgae for biodiesel production. *Fuel* 128, 46-52.
- Haass, D. & Tanner, W., 1974. Regulation of hexose transport in *Chlorella vulgaris*. *Plant Physiol.*, 53, 14-20.
- Halim, R.; Danquah, M. K.; Webley, P. A., 2012. Extraction of oil from microalgae for biodiesel production: A review. *Biotechnol. Adv.*, 30, 709-732.
- Ho S., Chen C., Lee C., Chang J., 2011. Perspectives on microalgal CO₂ -emission mitigation systems — A review *Biotechnol. Adv.*, 29, 189-198
- John, R.P., Anisha, G.S., Nampoothiri, K.M., Pandey, A., 2011. Micro and macroalgal biomass: a renewable source for bioethanol. *Bioresource Technol.* 102, 186-193.
- Kobayashi, M., Kakizono, T., Yamaguchi, K., Nishio, N. & Nagai, S., 1992. Growth and astaxanthin formation of *Haematococcus pluvialis* in heterotrophic and mixotrophic conditions. *J. Ferm. Bioeng.*, 74, 12-20.
- Medina, A. R.; Grima, E. M., 1998. Downstream processing of algal polyunsaturated fatty acids. *Biotechnol. Adv.*, 16, 517-580.
- Miaoa X., Wu Q., 2006. Biodiesel production from heterotrophic microalgal oil. *Bioresource Technol.* 97, 841-846.
- Ogawa, T., Aiba, S., 1981. Bioenergetic analysis of mixotrophic growth in *Chlorella vulgaris* and *Scenedesmus acutus*. *Biotechnol. Bioeng.*, 23, 1121-32.
- Pagnanelli F., Altimari P., Trabucco F., Toro L., 2014. Mixotrophic growth of *Chlorella vulgaris* and *Nannochloropsis oculata*: influence of glucose and nitrate concentration, *J. Chem. Technol. Biotechnol.* 89, 652-661
- Radakovits, R., Jinkerson, R.E., Darzins, A., Posewitz, M.C., 2010. Genetic engineering of algae for enhanced biofuel production. *Eukaryotic Cell* 9, 16.
- Richmond A., 2004. *Handbook of microalgal culture: Biotechnology and Applied Phycology*. Wiley-Blackwell.
- Singh, J.; Gu, S., 2010. Commercialization potential of microalgae for biofuels production. *Renew. Sustain. Energy Rev.*, 14, 2596-2610.
- Tanner, W., 1969. Light-driven active uptake of 3-O-methylglucose via an inducible hexose uptake system of *Chlorella*. *Biochem. Biophys. Res. Comm.*, 36, 278-83.
- Vanthoor-Koopmansa M., Wijffelsa R. H., Barbosa M. J., Eppink M. H.M., 2012. Biorefinery of microalgae for food and fuel. *Bioresource Technol.* 135, 142-149.
- Yen H., Hu I., Chen C., Ho S., Lee D., Chang J.. 2013. Microalgae-based biorefinery –From biofuels to natural products Hong-Wei. *Bioresource Technol* 135, 166-174
- Yessie W. Sari, Wilhelmus J. Mulder, Johan P. M. Sanders and Marieke E. Bruins, 2015. Review: Towards plant protein refinery: Review on protein extraction using alkali and potential enzymatic assistance. *Biotechnol. J.* 2015, 10, 1138-115
- Zhang B., Wang K., Hasjim J., Li E., Flanagan B.M., Gidley M.J., Dhital S..2014. Freeze-Drying Changes the Structure and Digestibility of B-Polymorphic Starches. *J. Agric. Food Chem.* 62, 1482-1491.