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ACHIEVEMENTS AND PERSPECTIVES IN HETERO- AND MIXOTROPHIC CULTURING OF MICROALGAE

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This work presents the experimental results obtained in the hetero- and mixotrophic culture of *Chlorella vulgaris*, a microalga belonging to the Chlorophyceae class. Objective of the study was evaluating the capability of the microalga to accumulate lipids, prospectively usable either in biodiesel production or in the extraction of nutraceuticals. Our study shows that *C. vulgaris* can increase its lipid content from ~15% (balanced-, autotrophically-grown biomass) to more than 50%, and highlighted the difference between the type (i.e., polar or nonpolar) of the lipids accumulated upon nitrogen and phosphorus starvation. An apparent interaction between diel rythms and glucose uptake was also suggested, together with its implications on productivity.

1. INTRODUCTION

The versatility of microalgae makes them extremely promising organisms: they can be used to fix CO_2, and prospectively treat gaseous effluents, accumulate lipids for biodiesel production, produce phyto-metabolites, and perform phytodepuration.
Most microalgae feature a photoautotrophic metabolism, whereby they trap light energy as the energy source and assimilate CO_2 by fixing it in the Calvin cycle and then converting it into carbohydrates; some microalgae can also use organic substrates instead, if they are present, as carbon, reducing power, and energy sources, i.e., they are *amphitrophic*. When both light and organic substrates are available, this latter group exhibits a mixed metabolism (mixotrophy). Some strains of *Chlorella* sp., *Dunaliella salina* and *Arthrospira platensis* (*Spirulina*) are amphitrophic but their growth does not benefit from mixotrophy; some other species of *Chlorella* and *Hematococcus pluvialis* do. However, while the production of microalgal biomass of these latter could receive a boost by mixotrophy (Lee, 2001), many practical hurdles hinder this deployment at the industrial scale—such as axenic operation, and supply of a low cost carbohydrate feed. For this latter problem pretreated lignocellulosic materials might offer opportunities (Hawkins, 1999) not devoid of concerns (Yu et al, 1990).
Chlorella sp. is produced by many companies around the world, with a nutraceutical target and small productions. The most important substance in *Chlorella* sp. is β-1,3-glucan, with various preventive and health promoting effects (Spolaore et al., 2006). *Chlorella* sp., moreover, is also an oleaginous microalga, i.e. it can accumulate more than 20% lipids, mostly C18:1, C16:0 and C18:3 (*C. vulgaris*, Wynn and Ratledge, 2006). Polyunsaturated fatty acid (PUFA) content and type discriminate the suitability for nutraceutical rather than biodiesel; *Chlorella* sp. Single Cell Oil (SCO) (or whole fatty cells) has therefore mostly been advocated for this latter (Miao and Wu, 2006).
This work presents the experimental results obtained in the heterotrophic culture of *C. vulgaris*, a freshwater microalga belonging to the Chlorophyceae class. Objective of the study was evaluating the capability of the microalga to accumulate lipids, and discuss opportunities for the production of biodiesel fuel or nutraceuticals by resorting to its hetero- and mixotrophic culture.

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2. MATERIALS AND METHODS

2.1. Media

Cultures A were based on a revisited Domingues et al. (2002) medium modified in its N, P and Fe^{3+} content according to the starvation model under test. Cultures B and C were based on Shi et al. (1997) medium, supplemented with olive oil mill wastewater (OOMW). OOMW was supplied by the "Calvi Risorta" (Italy) mill, stored refrigerated until conditioning (centrifugation, filtration, and boiling for 30') and use.

2.2. Biomass

A commercial strain of *Chlorella vulgaris* was used and continuously propagated photoautotrophically in indirect sunlight on a Shi (1997) medium.

2.3. Analytical Methods

Glucose. Glucose was determined by the Miller method (Miller, 1959) for reducing sugars. *Ammonium*. Ammonium concentration was determined by the Nessler method. *Polyphenols*. Polyphenols were measured by the Folin-Ciocalteau method (Singleton and Rossi, 1965) using gallic acid as standard and by gas chromatography with mass spectrometric detector (GC-MS) after derivatisation as described by Zafra et al. (2006). *Lipids*. Lipids analysis consisted in lipids extraction and gravimetric lipids quantitation. The former was carried out by the sequential application of a modified Ruiz et al. (2007) extraction method, followed by the ISO 659 extraction method. Specifically, the sample was treated with 100 ml of 1:1 dicloromethane:methanol solution for 15' in an ultrasonic bath, then for 2 h in an orbital shaker, thus dissolving all cellular lipids. The solids were then separated from the solution by vacuum filtration. The solution was rotoevaporated and total lipids were determined gravimetrically. The dried sample was then resuspended in hexane, agitated for 1 h in an orbital shaker and centrifuged to separate mucilages. By adding 1 ml of distilled water and a few drops of phosphoric acid phospholipids were insolubilised. The mixture was then allowed to settle, after which half of the added solution was carefully withdrawn from the surface. The withdrawn mixture was rotoevaporated for 30' and the remaining substance was weighed as nonpolar lipids. From the two, the lipid concentration was calculated. *Suspended solids*. Standard solid content analyses (TSS) for wastewater treatment systems were performed according to APHA–AWWA–WPCF (1992). *Cell count*. Cell count was performed by a cell counting chamber with Burker ruling.

2.5. Experimental Facility

The experimental runs described were carried out in a triple-vessel (1-l each) bubble-column photobioreactor (PBR) (Fig. 1 *left*) and in a 2-l general-purpose, mechanically stirred fermenter covered by aluminum sheets (Biostat B, Braun Biotech, Fig. 1 *right*).

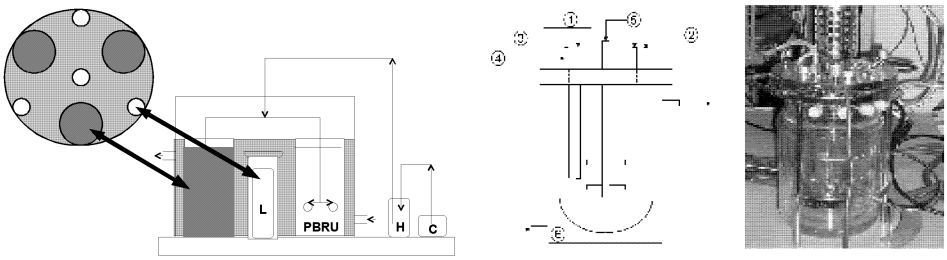

Figure 1. Equipment used in the experimentation. Left (a): Bubble-column photobioreactor; right (b): general-purpose, mechanically agitated fermenter .

2.6. Design of Experiments

Run Set A. Initially, the short-term heterotrophic lipid accumulation on glucose by *C. vulgaris* from a photoautotrophic culture was carried out: under N limitation (N-lim), N deprivation (N-dep), simultaneous N-lim and P-dep and, after Liu et al. (2008) finding, under a Fe^{3+}-supplemented, N-lim, P-dep medium. *Run Set B*. Three heterotrophic cultures at 5, 10 and 15% OOMW (upper limit adopted after Sánchez et al., 2001) were set-up to highlight short term growth on OOMW. *Run Set C*. A long-term mixotrophic growth and lipid accumulation was carried out on OOMW at time-varying concentrations of this latter.

3. RESULTS AND DISCUSSION

Run set A was aimed at investigating the time profile of glucose uptake during lipid accumulation by *C. vulgaris* and the ultimate lipid load exhibited by the biomass. Following starvation in a key nutrient, the cells convert the excess carbon in the growth medium into storage lipidic materials. In N limitation, the biomass takes up glucose more than doubling its total content of lipids from the initial (photoautotrophically grown) biomass (A1,2-PA to A1 in Table 1). It does not, however, substantially increase the nonpolar fraction. Conceivably, in shortage of nitrogen and in the presence of phosphorus, *C. vulgaris* anticipates future balanced growth opportunities by maximising membrane lipids. This explanation is confirmed by the results of run A2, carried out in the absence of nitrogen, where the fraction of polar lipids accumulated increases. Noting this, it seemed natural to investigate *C. vulgaris* behaviour in the absence of either nutrient. It can be seen that, although the total amount of lipids is slightly lower, most of them are actually tryacylglycerides, thus suitable for biodiesel use. In run A4 the biomass became leaner in total lipids, but mostly owing to the polar lipids decrease. Finally, our findings in the presence of Fe^{3+} in the medium (A5), advocated by Liu et al. (2008) for faster biomass growth and increased lipid accumulation, suggest that although total lipids are maximised, non polar lipids suitable for biodiesel production actually decrease.

The time profile of residual glucose concentration in the medium deviates abruptly (Figure 2) overnight, independently from the actual exposition to light, in some (A2 and A4) but not in other (A1) runs; in run A3 this behaviour can be observed during the first, but not during the second “night”, a surprising behaviour given the light shielding of the bioreactor. Possible marginal light leaks to the interior part of the photobioreactor (e.g., through the glass nozzles) causing occasional different overnight behaviour in A1—A3 (glucose uptake observed or not) prompted for double light insulation during A4. The observable periodicity is 12h:12h, corresponding to the light:darkness ratio used in our photoautotrophic photobioreactor (different from the “natural” 16h:8h).

Seemingly, this phenomenon is not mentioned in the literature. It could be questioned whether it is an intrinsic 24-h biological rhythm (i.e. circadian) or it is symply synchronised to a (memorised) light:darkness forcing (i.e. diel). This periodicity might perhaps decay after several days (similarly to what Ral et al., 2006, observed for starch accumulation in *C. reinhardtii*). Given that a nightly stop in glucose uptake would jeopardise plant capacity, countermeasures to it should be investigated.

The results of run set B (Figure 3, *left*) highlighted that unacclimatised *C. vulgaris* could not grow above the 5% OOMW concentration limit. At 5%, however, the microalga exhibited a substantial growth after a 5-day lag and was able to degrade 2/3 of the polyphenols load of the culture medium (300 ppm), as pointed out by GC-MS analysis of the terminal supernatant carried out against an equally air-sparged reactor devoid of biomass (results not shown), while the analysis by the Folin-Ciocalteau method had exhibited minimal terminal degradation and an inconsistent time profile.

The mixotrophic growth and lipid accumulation on OOMW was carried out in the multiple-bubble column photobioreactor. A photoautotrophic culture of *C. vulgaris* was set up and run for 10 days, after which OOMW was added at 2% by volume.

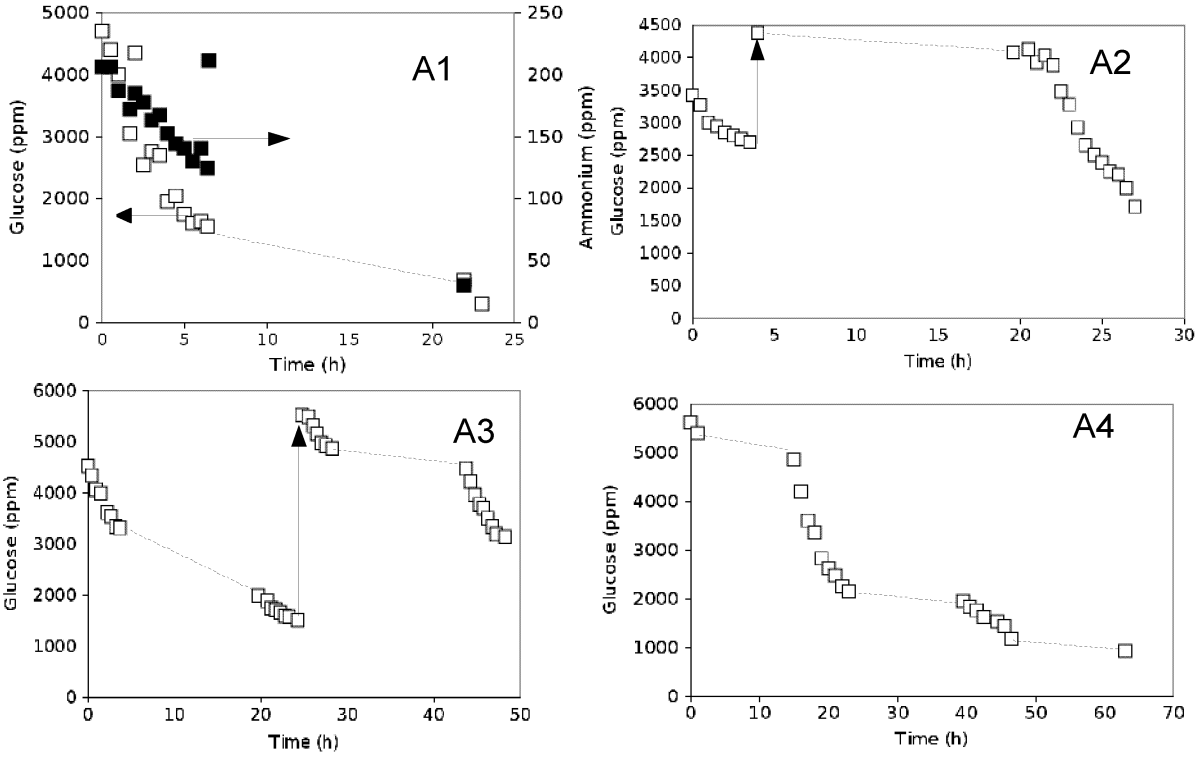

Figure 2: Time profile of glucose uptake during heterotrophic lipid accumulation by C. vulgaris in various culture conditions. Top left: N-deficient; top right: N-deprived; bottom left: N- and P-deprived; bottom right: N-limited and P-deprived.

After 20 days, 50% of the culture was withdrawn and replaced with fresh growth medium and OOMW proportion was adjusted to 5%. The algal biomass contained in the separated suspension was recovered and analysed for lipids (C1,2-1 in Table 1); this was done for all subsequent samples and for the whole culture at the end of the experiment. After 10 more days, again, 50% of the culture was withdrawn (C1,2-2) and replaced with fresh growth medium and OOMW. On the 50th day the whole culture was interrupted.

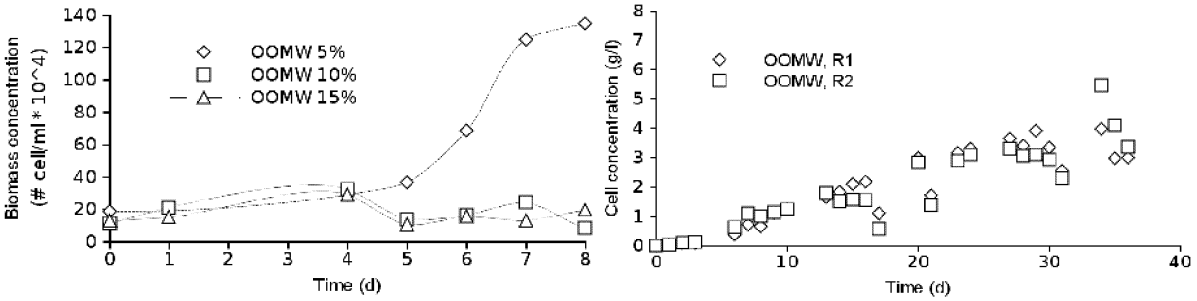

Figure 3: Time profile of microalgal growth during Run Set B (left) and C (right).

Biomass growth in Run Set B was mixotrophic by design, in that the culture was exposed to light. However, culture medium of B runs was very dark due to the presence of OOMW, so that the biomass presumably grew heterotrophically in most of the PBR volume. Biomass growth was mostly linear during the run, a finding suggestive of growth under some limitation of which, in the the absence of further information, either the photoautotrophic or the heterotrophic metabolism may be responsible. Given medium darkness, photoautotrophic growth was likely severely light-limited, so that either the heterotrophic growth was even slower, or it was equally subjected to some substrate limitation.

Table 1. Lipid content of the microalgae from Run Sets A and C. PA: photoautotrophic.

Run Set A	Total (% dw)	Neutral	Run Set C	Total	Neutral
A1,2-PA	16.5	3.0	C1	3.6	—
A1 (*N-lim*)	35.0	3.7	C2	15.2	—
A2 (*N-dep*)	51.8	11.9	C3	19.4	11.6
A3,4,5-PA	20.0	12.5	C4	25.2	5.1
A3 (*N,P-dep*)	49.7	46.3			
A4 (*N-lim, P-dep*)	44.5	43.1			
A5 (*as A4, +Fe*)	58.9	44.3			

4. CONCLUSIONS

Our study showed that *C. vulgaris* is able to increase its lipid content from ~15% to more than 50% and demonstrated the fundamental difference between the type (i.e., polar or nonpolar) of lipids accumulated by *C. vulgaris* upon nitrogen and phosphorus starvation. The possibility of using OOMW as a growth medium was confirmed and some lipid accumulation on OOWM, although lower than on glucose, was assessed.

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REFERENCES

APHA–AWWA–WPCF, 1992. Standard methods for the examination of water and wastewater. APHA, Washington, DC.

Domingues, F. C.; Queiroz, J. A.; Cabral, J. M.; Fonseca, L. P., 2000. The influence of culture conditions on mycelial structure and cellulase production by *Trichoderma reesei* Rut C-30.. Enz Microb Technol 26 (5-6) 394—401.

Hawkins, R. L., 1999. Utilization of Xylose for Growth by the Eukaryotic Alga, *Chlorella.* Curr Microbiol 38 (6) 360—363.

Lee, Y. K., 2001. Microalgal Mass Culture Systems and Methods: Their Limitation and Potential J. Appl. Phycol. 13 (4) 307—315.

Liu, Z. Y.; Wang, G. C.; Zhou, B. C., 2008. Effect of iron on growth and lipid accumulation in *Chlorella vulgaris.*Biores Technol, 99 (11) 4717—4722.

Lüning, K., 2006. Algal culturing techniques. Elsevier Acad. Press, Burlington, Mass.

Miao, X., Wu, Q., 2006. Biodiesel production from heterotrophic microalgal oil. Bioresource Technology 97 (6) 841—846.

Miller, G. L., 1959, Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. An Chem 31 (3) 426—428.

Ral, J. P.; Colleoni, C.; Wattebled, F.; Dauvillee, D.; Nempont, C.; Deschamps, P.; Li, Z.; Morell, M. K.; Chibbar, R.; Purton, S.; d'Hulst, C.; Ball, S. G., 2006. Circadian Clock Regulation of Starch Metabolism (...) in *C. reinhardtii.* Plant Physiol 142 (1) 305—317.

Ruiz, N., Dubois, N., Wielgosz-Collin, G., du Pont, T. R., Berge, J.-.P, Pouchus, Y. F., Barnathan., G., 2007. Lipid content and fatty acid composition of a marine-derived *T. longibrachiatum* strain (...). Proc Biochem 42, 676—680.

Sánchez, S.; Martínez, M. E.; Espejo, M. T.; Pacheco, R.; Espinola, F.; Hodaifa, G., 2001. Mixotrophic culture of *Chlorella pyrenoidosa* with olive-mill wastewater as the nutrient medium. J Appl Phycol 13 (5) 443—449.

Shi, X.M.; Chen, F.; Yuan, J.P.; Chen, H., 1997. Heterotrophic production of lutein by selected *Chlorella* strains. J Appl Phyc 9 (5) 445—450.

Singleton, V. L.; Rossi, J. A., 1965. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. Am J Enol Vitic 16 (3) 144—158.

Spolaore, P.; Joannis-Cassan, C.; Duran, E.; Isambert, A., 2006. Commercial applications of microalgae. J Biosci Bioeng 101 (2) 87—96.

Yu, S.; Forsberg, Å.; Kral, K.; Pedersén, M., 1990. Furfural and Hydroxymethylfurfural inhibition of growth and photosynthesis in *Spirulina*. Eur J Phycol 25 (2) 141—148.

Wynn, J. P.; Ratledge, C., 2006. Microbial Production of Oils and Fats, in: Food Biotechnology (Ed. Shetty, K. et al.). CRC Press, 443—472.

Zafra, A.; Juárez, M. J. B.; Blanc, R.; Navalón, A.; González, J.; Vílchez, J. L., 2006. Determination of polyphenolic compounds in wastewater olive oil by gas chromatography—mass spectrometry. Talanta, 70 (1) 213—218.