

Fungal Biomass-Based Production of Lipids for Biodiesel Synthesis

D'Urso, A., Bubbico, R., Scarsella, M., Bravi, M.*

Dipartimento Ingegneria Chimica Materiali Ambiente; Sapienza – Università di Roma
via Eudossiana 18; I-00184 Rome (Italy)

*marco.bravi@uniroma1.it

Lipids for biodiesel fuel production are ordinarily being extracted from terrestrial plants, while alternative, fast-growing, lower cost sources are being sought in microbial sources. Microbial lipids can be extracted from many sources, but economical and technical issues mostly negate the possibility to use them in low oil cost destinations. The present paper revises the problems connected with low-cost microbial lipid production and investigates the use of fungi in the production of lipids by growing them on waste materials.

1. Introduction

Biodiesel fuels, short-chain esters of fatty acids, are generally obtained by transesterification of triacylglycerides (TAGs) derived from vegetable oils or animal fats. The most recent trend in biodiesel production deals with using specialised oil-rich crops (such as rapeseed) and devoting an increasing amount of land to their cultivation; this strategy, along with not being quantitatively significant, is postulated to create a competition for the cultivable land area with basic crops of our diet and economy and cause cost fluctuations of these latter. A minimally intrusive biofuel production policy should turn to waste materials as potential lipid sources.

Many microorganisms accumulate cytosolic lipid bodies, including microalgae, protozoans, fungi and prokariotes (Murphy et al., 2005). In the present work, the potential use of microbial biomass grown on agro-food waste is first surveyed to highlight potential contributions to the production of lipids for conversion into biodiesel. The second purpose of this work is presenting the results of a semi-model investigation of the production of fatty acids to be converted into biodiesel fuel by means of moulds. In particular, in this work, *Trichoderma viride* is being considered.

Trichoderma spp. are able to store lipids (Serrano-Carreón et al., 1992); *T. viride*, in particular, tolerates biorecalcitrant compounds and strongly acidic environments, thus envisaging interesting feeding scenarios based on environmentally critical wastewaters.

This investigation was performed by characterising the behavior of the fungus in a synthetic liquid growth medium containing glucose or lactose and on a fed-batch bioreactor where a culture of *T. viride* was grown on olive oil mill wastewater (OOWM), chosen because of its large production in South-European countries and its highly pollutant character.

2. Lipids from microorganisms

Microorganisms capable of accumulating lipids to more than 20% of their dry weight, and therefrom termed *oleaginous* (Ratledge and Wynn, 2002) can be used to extract *microbial* lipids, investigated and exploited as alternative sources of oils and fats for human consumption and, increasingly, for renewable energy production. Basic and applied research has covered fungal microorganisms, prokaryotes and microalgae.

Microbial lipids are not easy to produce economically. If fungi are cultured on glucose or sucrose derived from agricultural crops, the cost of turning one agricultural commodity into another at the relevant yield factor ($Y_{oil/sugar} \sim 0.2$) and their respective costs ($cost_{sugar}:cost_{oil} \sim 1:4$; Wynn and Ratledge, 2006) rule their production process out. Making microbial lipid production profitable requires either that price of the produced oil exceed that of commodity supplies and/or that a free carbon source is available. Currently, while the former opportunity is behind some running processes, all of them are being actively investigated.

Free carbon is available from two sources: inorganic, such as CO_2 from the atmosphere, or from combustion emissions, and organic, such as waste materials, in aggregated (waste solids) or dissolved state (wastewaters). High-value oily materials rich in nutritionally significant polyunsaturated fatty acids (PUFAs) are generally investigated for nutraceutical uses, thus requiring GRAS (Generally Recognised As Safe) practices: a suitable pick of microorganisms, raw materials, equipment and procedures and (mostly) ruling out waste materials. On the other hand, strict standards are (mostly) not required for such low value end-products as biofuels, which can benefit from free inorganic carbon from the atmosphere and free organic carbon from many by-products of the agro-food industry; on the other hand, however, the very same richness in PUFAs which makes microbial lipids nutritionally interesting raises concerns about the oxidative stability of the biodiesel fuel produced therefrom (Falk and Meyer-Pittroff, 2004).

Inorganic carbon sources provide photolithoautotrophic organisms with reducing equivalents at the same time they supply them carbon, energy being provided by (sun)light, thus leading to a net subtraction of CO_2 ; conversely, organic carbon sources provide chemoorganoheterotrophic organisms supplying energy, reducing equivalents and carbon at a time, thus leading to a net release of CO_2 . Sometimes, the two metabolic behaviours coexist (mixotrophic metabolism), generally yielding an overall improved growth rate of the microbial biomass.

Lipid accumulation in the cells of microorganisms is a stress-induced response to the shortage of some nutrient other than the carbon source—usually nitrogen. Following starvation in a key nutrient, the cells enter a phase in which the excess carbon in the growth medium is converted into storage lipidic materials.

A typical profile for the accumulation of lipid in an oleaginous microorganism is shown in Figure 1. This shows that lipid accumulation in a microbial cell only begins when nitrogen is exhausted from the medium.

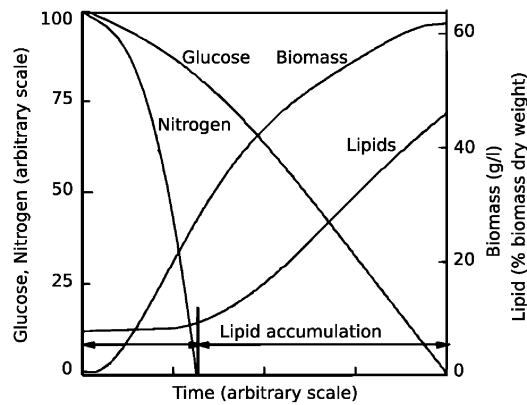


Figure 1: Schematic illustration of the time profile of lipid accumulation in an oleaginous microorganism (Wynn and Ratledge, 2006).

In heterotrophic cultures, the growth medium has to be formulated with a high C:N ratio to ensure that nitrogen is exhausted while other nutrients, including carbon, remain in excess. Usually a 40 to 50:1 carbon-to-nitrogen ratio (C:N) is used, although the optimal value needs to be determined for each individual organism. In order to maximise the cell biomass concentration and reduce the volume of treated suspension for the separation of the developed biomass, the nitrogen and carbon sources concentration may need to be increased while keeping their proportion; this enables a balanced growth phase to continue until the maximum biomass density that the fermentor can sustain is reached before the lipid accumulation phase begins.

Restoring the availability of the missing nutrient reverts the lipid accumulation mechanism, so that cellular materials are assembled again.

Similarly, photoautotrophic cultures of microalgae tend to accumulate lipids in nitrogen-limited media (Guschina et al., 2006).

A number of microalgae can supplement photoautotrophic metabolism with heterotrophic metabolism, thus exhibiting mixotrophy. True facultative mixotrophic microalgae (i.e., able to grow in either photoautotrophic and heterotrophic conditions), such as *Chlorella* spp., *Haematococcus pluvialis*, *Scenedesmus acutus* and *spirulina platensis* can be effectively grown on glucose and acetate. Mixotrophically grown *Chlorella* or *Haematococcus* grow faster than either pure photoautotrophically or heterotrophically grown biomass belonging to the same genera (Lee, 2001), exhibiting interaction coefficients between the photoautotrophic and heterotrophic metabolism (Ogbonna et al., 2002) lower than unity.

In true facultative mixotrophs, heterotrophic growth has been demonstrated by Miao and Wu (2006) to yield an increased lipid accumulation compared to photoautotrophic growth.

Most “free” carbon sources suitable for heterotrophic or mixotrophic cultures do not possess the required C:N ratio and/or are not concentrated enough to produce a high-

density culture. Exceptions exist, but may not be immediately ready for culture use—such as lignocellulosic residues, which need to be preliminarily hydrolysed to deliver their readily metabolisable sugars content.

Table 1 contains a small excerpt of microbial lipid sources from the open literature falling in all of the classes named above, and providing an introduction for the presented experimental results.

3. Lipids from *Trichoderma* spp.

3.1 Rationale for an Investigation

The rationale behind the idea of using fungi to store lipids lies in their general ability of growing on wood and other lignocellulosic materials thanks to a pool of intra- and extracellular phenyl-, glucan- and xylan-degrading or modifying enzymes (Hammel, 1997): *T. viride* (phylum Ascomycota), in particular, is a cellulase high producer (Domingues et al., 2000) which is able to grow in strongly acidic media (Brown and Halsted, 1975) and thus survives in acid wastewaters. The authors of this paper also highlighted the ability of *T. viride* to grow on phenolics-rich wastewaters in model growth media containing concentrated gallic acid in short- (D'Urso et al., 2007a) and long-duration experimental runs (2008a), olive oil mill, distillery and cork processing wastewaters in short duration runs (2007b) and olive oil mill wastewaters in long-duration runs (2008b).

Olive oil production delivers large amounts of wastes in liquid, dilute or concentrated suspension and solid state. Liquid and dilute suspensions (OOMWs) are especially hard to dispose of, due to their high organic load (biochemical and chemical oxygen demand up to 100 and 220 g l⁻¹), their content of polyphenols, tannins and suspended solids and to their acidity (Annesini & Gironi 1991). While polyphenolic compounds exhibit a limited toxicity and biodegradability, tannins and simple phenolic compounds are highly toxic but biodegradable (Hamdi, Garcia & Ellouz, 1992). OOMW features a C:N ratio in the order of 10:1—20:1. Two-phase continuous centrifugation process feature increased processing capacity and extraction yield, and decreased water consumption and wastewater production (Alba, Hidalgo, Martínez, Ruiz, Moyano & Borja, 1995) exhibit a C:N ratio of 45±10:1.

High COD wastewaters are an interesting kind of carbon source, fitting nicely the need of low-cost microbial lipids production. In principle, these wastewaters can be used to feed any kind of biomass which is able to efficiently metabolise the sugars contained therein; in practice, they can end up hosting competing microorganisms unless the microbial process is run axenically, which would offset the economic benefits of a free carbon source. In this respect biorecalcitrant, low pH, high COD and high C:N ratio wastewaters are indeed a “special” kind of “free” carbon source, in that they would represent a unique selective environment for microorganisms (mostly) permitting a low-axenicity process conduite or, in the worst case, only requiring a mild pasteurisation before inoculation of the target microorganism.

Table 1. Some oleaginous microorganisms and their main lipids-production related performance (b.: batch...; Ref. key indicates first author-year; T.w.: *this work*).

| Species | Substrate | Growth rate (h ⁻¹) | Biomass (g _B l ⁻¹) | Lipid conc. (g _L g _B ⁻¹) | Lipid prod. (g l ⁻¹ h ⁻¹) | Lipid type | Y _{L/S} | Lipid Unsat. | Ref. |
|---|------------------------------|-----------------------------------|--|---|---|---------------|------------------|-----------------|--------|
| <i>Fungi</i> | | | | | | | | | |
| <i>Candida curvata</i> | glucose | | 13.5 | | 0.16 | | 0.29 | | W06 |
| <i>Candida curvata</i> | lactose | | 18 | | 0.22 | | 0.31 | | W06 |
| <i>Candida curvata</i> | xylose | | 15 | | 0.27 | | 0.37 | | W06 |
| <i>Apiotrichium curvatum</i> | whye | | 20 | | 0.38 | | 0.36 | | W06 |
| <i>Trichoderma viride</i> | glucose | 0.012 | 2.2 | 0.162 (b.) | | | | | (T.w.) |
| <i>Trichoderma viride</i> | glucose | 0.009 (b.) | | 0.35 (b.) | | TAG | 0.055 | 1.17 (b.) | (SC92) |
| <i>Trichoderma viride</i> | lactose | 0.012 | 2.0 | 0.128 (b.) | | | | | (T.w.) |
| <i>Trichoderma viride</i> | sucrose | | | | | TAG | | | (SC92) |
| <i>Trichoderma viride</i> | xylose | | | | | TAG | | | (SC92) |
| <i>Trichoderma viride</i> | OOWM | 0.012 | 20.0 | 0.074 (b.) | | | | | (T.w.) |
| <i>Rhodotorula toruloides</i> | | 0.13 (f.b.) | 151 (f.b.) | 0.48 (f.b.) | | TAG | 0.26 | 0.84 (f.b.) | L07 |
| | | 0.135 (b.) | 18.6 (b.) | 0.68 (b.) | | TAG | 0.23 | | |
| | | | | 0.72 | | TAG | | 0.67 | |
| <i>Rhodotorula glutinis</i> | glucose | | 10.9 | 0.35 | | TAG | 0.11 | 0.85 | P07 |
| <i>Mortierella isabellina</i> | lactose | | 9.5 | 0.37 | | TAG | 0.14 | 0.83 | P07 |
| <i>Mortierella isabellina</i> | glucose | | 9.2 | 0.35 | | TAG | 0.11 | 0.90 | P07 |
| <i>Cunninghamella echinulata</i> | lactose | | 0.6 | 0.05 | | TAG | 0.01 | | P07 |
| <i>Cunninghamella echinulata</i> | | 0.516 | 26.4 | | 40.5 | TAG | | | |
| <i>Mortierella alpina</i> (DSM, W.-A. proc's) | | | | 0.36 | 0.08 | TAG | 0.25 | 1.42 | |
| <i>Yarrowia lipolytica</i> | | 0.21 (b.) | 8.1 (c.) | 0.43 | 0.12 | TAG | 0.43 | 0.93 | |
| <i>Yarrowia lipolytica</i> | Ind'l glycerol | | | | | | | | |
| <i>Algae</i> | | | | | | | | | |
| <i>Chlorella vulgaris</i> (true mixotroph) | CO ₂ (Photoaut.) | 0.110 | | | | TAG | | | O81 |
| <i>Chlorella vulgaris</i> (true mixotroph) | glucose (Het.) | 0.98 | | | | TAG | | | O81 |
| <i>Chlorella vulgaris</i> (true mixotroph) | CO ₂ + glc (Mix.) | 0.198 | | | | TAG | | | O81 |
| <i>Chlorella protothecoides</i> | CO ₂ (Photoaut.) | 0.015 | | 0.15 | | TAG | | | X06 |
| <i>Chlorella protothecoides</i> | CPH (Het.) | 0.015 | 3.9 | 0.55 | | TAG | | | X06 |
| <i>Botryococcus braunii</i> | CO ₂ (Photoaut.) | 0.014 | | 0.27–0.86 | | HC | | | D05 |

3.2 Materials and Methods

The *T. viride* strain, originally from Centraalbureau voor Schimmelculters (CBS) of Baarn (Holland), was obtained from Prof. Lo Curto (University of Messina, Italy), conserved as a pure culture on glucose agar medium and carefully propagated therefrom in the growth medium.

3.3 Analytical Methods

The lipid analysis was carried out following Riuz et al. (2007) using two extractive solutions (S1 and S2) of dichloromethane and methanol 1:2 and 2:1 volume fractions. The biomass was first orbitally shaken; then, after centrifugation, the suspension was added to 50 ml of S1 and agitated for 2 hours at 150 rpm at 25 °C. The liquid fraction was recovered by filtration and stored while the solid fraction was extracted in the same way with 50 ml of S2 and the liquid recovered, again, by filtration. The two extracts were mixed and the solvents were rotoevaporated, leaving the lipidic phase behind, which was dried for 24h in a drying oven. Standard solid content analyses (TSS) for wastewater treatment systems were also performed according to APAT and IRSA-CNR (2004). From the two, the lipid concentration was calculated.

3.4 Experimental Runs, Results and Discussion

The suitability of using wastewaters as a growth medium for oil-storing microorganisms was evaluated on *T. viride* and three different media, constituted by a pool of nutrients and microelements (adopted after Domingues et al., 2000) and one substrate. Two model substrates (glucose and lactose) and olive oil mill wastewater were used.

The OOWM tests were carried out with treatment in mind, e.g. targeting COD reduction. The biomass was inoculated in raw OOWM (featuring a C:N ratio which was estimated to be ~10:1 and grown for one week. Then, 100 ml of concentrated biomass suspension were used to make the lipid extraction.

Model systems nutrients and microelements were supplemented with glucose and lactose so as to obtain a C:N ratio equal to 60. The growth medium was inoculated with 100µL of biomass and grew under agitation for 7 days at 150 rpm and 25°C with periodic biomass growth monitoring. Subsequently, the general lipid determination procedure was applied.

The results of the three runs are 7.4% (lipids on biomass dry weight), 16.2% and 12.8% respectively for the OOWM, glucose and lactose runs.

Serrano-Carreón et al. (1992) lipid accumulation tests on *T. viride* on C:N = 60 media using glucose and ammonium sulphate (matching our choice) as substrate and nitrogen source, respectively, attained ~13% lipid fraction after 7 days and 32% after 11 days. Clearly, lipid accumulation is accelerating after the first week of culture. Our culture was stopped after 7 days to match the time allowed for the OOWM treatment. The slightly lower lipid accumulation might be due to oxygen limitation. Lactose, apparently, leads to a lower lipid accumulation. The hydrolysis of the disaccharide may explain this delay; a comparable difference in lipid accumulation on glucose vs sucrose-fed cultures was indeed observed by the above Authors, and might require that the culture time be extended to reach comparable performance.

The OOWM culture exhibits a much worse lipid accumulation performance, and the question can be raised as to whether olive by-product treatment may be a suitable

opportunity to store lipids. The main reason is surely due to an unfavourable overall-C:N ratio; the second may be due to the carbon substrate distribution across several compounds, some of which are quickly metabolised, while others require some degradation before they can be metabolised, as observed before for disaccharides.

An answer to this question can be given if OOWM is combined (or replaced) by olive pomace, in that *T. viride* rapidly excrete a large amount of cellulase which, in the same time frame of lipid accumulation (some days), is able to degrade cellulose and might conceivably increase lipid accumulation.

4. References

- Alvarez, H., Steinbüchel, A. Triacylglycerols in prokaryotic microorganisms. *Applied Microbiology and Biotechnology* 60 (4) 367—376 (2002).
- Brown, D.E. and D.J. Halsted, 1975, The effect of Acid pH on the Growth of *Trichoderma viride*, *Biotech. and Bioeng.*, 17, 1199-1210.
- Brown, D. E., Hasan, M., Lepe-Casillas, M. and Thornton, A. J. Effect of temperature and pH on lipid accumulation by *Trichoderma reesei*. *Applied Microbiology and Biotechnology*, 34 (3), 335—339 (1990).
- Cohen, Z., Ratledge, C. (eds) *Single Cell Oils*. AOCS Press (2005).
- Dayananda, C., Sarada, R., Bhattacharya, S., Ravishankar, G. A. Effect of media and culture conditions on growth and hydrocarbon production by *Botryococcus braunii*. *Process Biochemistry* 40 (9) 3125—3131 (2005).
- Domingues, F.C., J.A. Queiroz, J.M.S. Cabral and L.P. Fonseca, 2000, The influence of culture conditions on mycelial structure and cellulase production by *Trichoderma reesei* Rut C-30, *Enzyme Microb. Technol.*, 26, 394–401
- D'Urso, A., Galimberti, M., Bravi, M., Fungal Biomass-Based Processing of Phenolics-Rich Wastewaters. Proc. 8th Intl. Conf. on Chem. and Proc. Engng., Ischia, June 24—27 (2007a).
- D'Urso, A., Russo, M., Bravi, M., Phenolic fraction removal by *Trichoderma viride*: an application on olive oil mill, distillery and cork processing wastewaters. Proceedings of Food and Agricultural Products: Processing and Innovations CIGR-III, Naples, (2007b).
- D'Urso, A., Gapes, D., Bravi, M., Fungal biomass activity on synthetic wastewater containing glucose, acetic acid and gallic acid. *This conference* (2008a).
- D'Urso, A., Gapes, D., Bravi, M., Bioremediation of Olive Oil Mill Wastewaters by Fungal (*T. viride*, strain 8/90) Sequencing batch reactor. *This conference* (2008b).
- Falk, O., Meyer-Pittroff, R. The effect of fatty acid composition on biodiesel oxidative stability. *European Journal of Lipid Science and Technology* 106 (12) 837—843 (2004).
- Guschina, I. A., Harwood, J. L. Lipids and lipid metabolism in eukaryotic algae. *Progress in Lipid Research*, 45 (2) 160—186 (2006).
- Hammel, K. E., 1997, Fungal degradation of lignin. In: *Driven by Nature: Plant Litter Quality and Decomposition*. Eds. Cadisch and Giller. CAB International, Wallingford.
- Hopwood, D. and de Vos, R. 20% by 2020: the way forward. *Refocus*, 8 (2), 22-25 (2007).

- Lee, Y. K. Microalgal mass culture systems and methods: Their limitation and Potential. *J. Appl. Phycol.* 13 (4) 307—315 (2001).
- Li, Y. H., Liu, B., Zhao, Z. B., Bai, F. W. Optimization of Culture Conditions for Lipid Production by *Rhodospiridium toruloides*. *Chinese Journal of Biotechnology* 22 (4) 650—656 (2006).
- Li, Y., Zhao, Z., Bai, F. High-density cultivation of oleaginous yeast *Rhodospiridium toruloides* Y4 in fed-batch culture. *Enzyme and Microbial Technology* 41 (3) 312—317 (2007).
- Miao, X., Wu, Q. Biodiesel production from heterotrophic microalgal oil. *Bioresource Technology* 97 (6) 841—846 (2006).
- Murphy, D. J. The biogenesis and functions of lipid bodies in animals, plants and microorganisms. *Progress in Lipid Research* 40 (5) 325—438 (2001).
- Ogbonna, J. C., Ichige, E., Tanaka, H. Interactions between photoautotrophic and heterotrophic metabolism in photoheterotrophic cultures of *Euglena gracilis*. *Applied Microbiology and Biotechnology* 58 (4) 532—538 (2002).
- Papanikolaou, S., Aggelis, G. Lipid production by *Yarrowia lipolytica* growing on industrial glycerol in a single-stage continuous culture. *Bioresource Technology* 82 1 43—49 (2002).
- Papanikolaou, S., Galiotou-Panayotou, M., Fakas, S., Komaitis, M., Aggelis, G. Lipid production by oleaginous Mucorales cultivated on renewable carbon sources. *European Journal of Lipid Science and Technology* 109 (11) 1060—1070 (2007).
- Ratledge, C., Wynn, J. P. The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. *Adv. Appl. Microbiol.* 51, 1—51 (2002).
- Ratledge, C., Fatty acid biosynthesis in microorganisms being used for Single Cell Oil production, *Biochimie*, 86 (11) 807—815 (2004).
- Ruiz, N., Dubois, N., Wielgosz-Collin, G., du Pont, T. R., Berge, J.-P., Pouchus, Y. F., Barnathan, G.. Lipid content and fatty acid composition of a marine-derived *Trichoderma longibrachiatum* strain cultured by agar surface and submerged fermentations. *Process Biochemistry* 42, 676—680 (2007).
- Serrano-Carreón, L., Hathout, Y., Bensoussan, M. and Belin, J.-M. Lipid accumulation in *Trichoderma* species. *FEMS Microbiology Letters*, 93 (2), 181—187 (1992).
- Suutari, M. Effect of growth temperature on lipid fatty acids of four fungi (*Aspergillus niger*, *Neurospora crassa*, *Penicillium chrysogenum*, and *Trichoderma reesei*). *Archives of Microbiology*, 164 (3), 212—216 (1995).
- Tomati, T., Various authors. Stato dell'Arte sul problema dell'utilizzazione dei reflui oleari. Ministero della Ricerca Scientifica e Tecnologica della Repubblica Italiana, Programma Nazionale di Ricerca "Reflui del Sistema Agricolo-Industriale", Sottoprogetto reflui oleari (2000).
- Woodbine, M. Microbial fat: Microorganisms as potential fat producers. *Prog. Ind. Microbiol.* 1, 179—245 (1959).
- Wynn, J. P., Ratledge, C., Microbial Production of Oils and Fats, in: Shetty, K., Paliyath, G., Pometto, A., Levin, R. (Eds.) *Food Biotechnology*, CRC Press, 443—472 (2006).