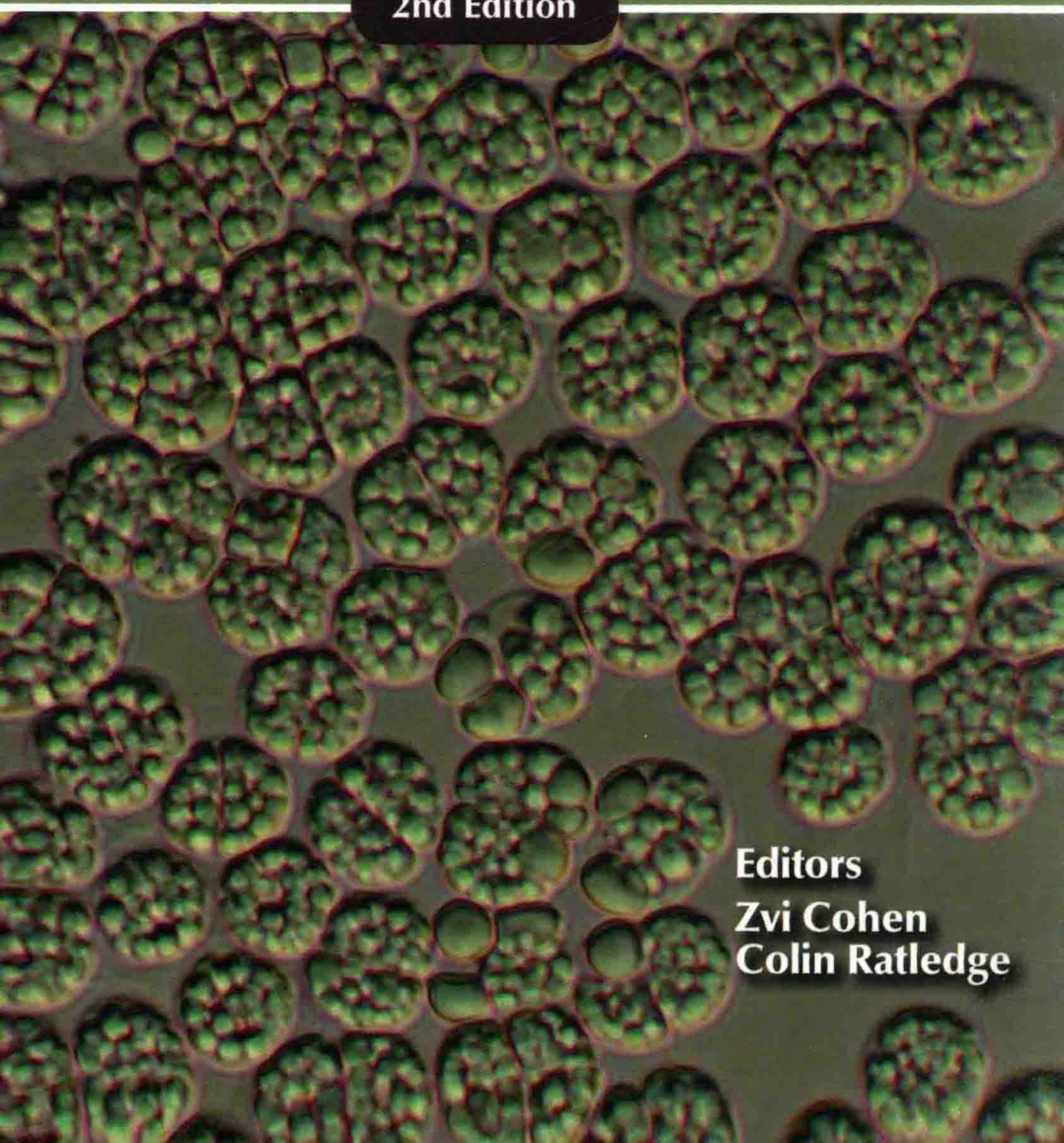
Single Cell Oils

Microbial and Algal Oils

2nd Edition



Editors
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Preface to the Second Edition

The pace of developments in the field of microbial oils and fats—the Single Cell Oils of the title—over the past 4 to 5 years has surprised us both. So much so, that a new edition of this book has had to be prepared to keep readers up to date. Of considerable interest and, to an extent, to our amazement have been the many commercial proposals since 2005 for developing microbial oils as sources of biofuels, particularly using photosynthetic algae. Some of these ideas appear very serious indeed with developments and investments being announced at very frequent intervals. Reports of these activities are now included in this new edition, together with considerations for developing the potential of bacteria for biofuel production. Biofuels can undoubtedly be produced from microalgae but the cost is highly problematic. Even with additional research and perhaps genetic manipulation to improve productivities and lower costs, we remain somewhat skeptical about the feasibility of producing such biofuels at a cost sufficiently low to be competitive with petroleum oils. However, our skills in gazing into the crystal ball to divine the future are somewhat limited. No one can tell what will be the price of petroleum oils in the years to come, let alone guess what levels of subsidies some governments might be prepared to pay for the dubious honor of being able to claim that they are using 'microbial or algal oils for fuel'. But, and we should not grumble too much, therefore, these activities are acting as tremendous stimuli for developing new ideas and interests in this subject. Indeed, this is the very type of stimulus that the entire subject of microbial oils has been lacking since its inception in the early decades of the 20th century.

Biofuels apart, what now can be regarded as the more 'traditional' areas for the uses of microbial oils—the production of the very long-chain polyunsaturated fatty acids (VL-PUFAs) for infant, adult, and animal nutrition—have also seen considerable advancement over the past 5 years. The application of genetic techniques for cloning and transforming oleaginous microorganisms into the production of novel PUFAs, or for producing ones previously not easily available either from animals or plants, has been successful; and chapters on the molecular breeding of yeasts and molds are now included in this second edition. The days of producing what have previously been described as 'designer' oils are clearly now with us. In addition, we have seen a much wider appreciation of the value of VL-PUFAs as essential dietary components by the general public and, from this, a realization that supplies of these oils from the traditional marine sources may not be sustainable nor, for many groups of people, acceptable, because of their animal origins. The presence of various pollutants that are concentrated in marine lipids further exacerbate the situation. Thus, there is now much more impetus from many companies around the world for producing

and exploiting microbial oils. These oils have gone from being academic curiosities to being minor commodity oils in their own right within a very short space of time.

Of course, everyone—even the editors—admit that microbial oils, particularly using nonphotosynthetic microorganisms, are expensive to produce because of the need for large-scale fermentation systems that involve the purchase of even larger amounts of sugar as fermentation feedstocks. If the key fatty acids could be produced by genetically modified plants, this would be a much cheaper route for obtaining them. But, in spite of well more than a decade of devoted research by a myriad of plant geneticists and even plant breeders, it has proved virtually impossible to produce plants that yield useful amounts of the key VL-PUFAs. Again, our crystal ball does not allow us to see the future in this area with any accuracy but it seems more than likely that at least another decade will have to pass before we see,the emergence of any plant-derived VL-PUFA. Thus, meanwhile, and perhaps in perpetuity, *Single Cell Oils* will command a key place in the marketplace. They may be expensive but they are produced by reliable, safe technologies that are not subject to the vagaries of the weather (or national politics)' do not involve the use of pesticides, insecticides, and herbicides; and are true organically derived materials. We believe that their position in the marketplace is now secure.

Zvi Cohen
Colin Ratledge
August 2009

Preface to the First Edition

Single cell oils (SCO) have come of age. They have become accepted biotechnological products fulfilling key roles in the supply of the major very long chain polyunsaturated fatty acids (PUFA), now known to be essential for infant nutrition and development. But their acknowledgment as being potential sources of oils and fats has been a slow process. Many critics in the early years of SCO doubted whether they could ever be produced at a reasonable price; even if they could, there were grave doubts as to whether SCO would be accepted by the general public. This was in spite of the "general public" having no apparent objection to consuming bacteria and yeasts as part of their everyday diet in the form of yogurts, cheeses, beers, and sourdough breads. When the product is good, the public will buy it; when the product is essential, the public will line up to buy it; and when our babies need the product, the line is likely to be a very long one indeed. SCO are the edible oils extracted from micro-organisms—the single-celled entities that are at the bottom of the food chain. The best producers with the highest oil contents are various species of yeasts and fungi with several key algae also able to produce high levels of nutritionally important PUFA. Interest in SCO, as they have now become known, stretches back for over a century. Attempts have been made to harness the potential of various organisms, especially during the two world wars, in order to produce much needed oils and fats. Attempts have also been made to produce substitute materials for some of the major oilseed crops and even to produce a superior type of cocoa butter material. But it has been their potential to produce PUFA that has now galvanized the current interest in these SCO as oils rich highly desirable fatty acids essential for our well being and not readily available either from plants or animals. This monograph has arisen from a symposium organized by David Kyle for the American Oil Chemists' Society in May 2003 that covered many of the ongoing projects in this area. It echoes two earlier conferences of the AOCS, the first in 1982 in Toronto and the second in Chicago in 1992, also organized by David Kyle. Over the intervening years, the position of SCO has become much more secure. Processes that were just "twinkles in the eye" in 1992 now exist as commercial realities; SCO production processes occur not only in the United States, but also in Europe, Japan, and China. Interest in them is widespread and the prospects of producing a complete range of PUFA is within our grasp. Whether the next decade or so will see SCO being overtaken by oils coming from genetically engineered plants, as has been predicted by some, will remain a tantalizing prospect. The future, as always, will be awaited with interest. In the meantime, SCO are here and available.

Zvi Cohen
Colin Ratledge
January 2005

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PART 1

Introduction and Overview

1

Single Cell Oils for the 21st Century

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Introduction

Single cell oils (SCOs) are defined as the edible oils obtainable from single celled microorganisms that are primarily yeasts, fungi (or molds), and algae (Ratledge, 1976). Bacteria, which are also single cell microorganisms, do not usually accumulate edible oils but instead tend to produce other storage materials, such as poly-beta-hydroxybutyrate. However, the possible uses of bacteria for producing lipids in sufficient quantities to be commercially useful are covered elsewhere in this book (see Chapter 14), but these uses are entirely for lipids as biofuels and would not be suitable for edible purposes. The edible oils that are produced by eukaryotic microorganisms (i.e., those that have a defined nucleus and are, therefore, distinct from prokaryotic bacteria having no defined nucleus) are similar in type and composition to those oils and fats from plants or animals. This chapter aims to provide an introductory overview to SCO and to show that current interest in their production and use has developed out of a long history of exploitating microorganisms as sources of oils and fats. Without these early endeavors, it is quite possible that none of the commercial SCO products currently on the market would have been developed as the basic understanding behind the exploitation of microbial oils would have delayed for several decades.

The key event that changed microbial oils from being more or less academic curiosities 20–30 years ago (see Ratledge, 1992) to becoming important nutraceuticals for adults, as well as infants, was the emergence of overwhelming evidence for the dietary significance of very long chain, polyunsaturated fatty acids (VL-PUFAs), coupled with the realization that there was no adequate or safe source from plants or animals. What were originally considered to be unusual microorganisms have turned out to be extraordinarily important, since they are still the only realistic sources of these oils. The belief that genetically modified plants would be created in a few short years to supply these essential VL-PUFAs and that SCOs would quickly be superseded by GM-oils has not been substantiated. Such sources of PUFAs appear as remote as ever, and, perhaps somewhat hopefully,

microbial oils may be expected to continue to be of economic importance for several decades to come.

The Early Years

There has been interest in microbial lipids for over 130 years (Nageli & Lowe, 1878) and in exploiting them as alternative sources of oils and fats for human consumption since the early decades of the 20th century. Paul Lindner, working in Berlin, Germany, appears to have been the first person to develop a small-scale process to make fat using a species of yeast then called *Endomyces vernalis* and currently known as *Trichosporon pullulans* (Lindner, 1922; Woodbine, 1959). The development of microorganisms as a source of oils and fats continued to escalate during the first four decades of the last century with a number of groups in various countries studying not only the process of lipid biosynthesis but also the factors influencing its accumulation. These early endeavors in microbial oil production were reviewed in considerable depth by Woodbine (1959), and this review remains possibly the most thorough one available covering the world-wide development of microbial oil production from its very inception through the mid-1950s.

The problem, though, was that the oils and fats produced by oleaginous species of yeasts and fungi (the groups of microbes that are the highest producers are referred to as the "oleaginous" species) were not significantly different from the oils and fats obtained from plant seeds. Since these microorganisms had to be grown in culture media that contained glucose or sucrose as a source of carbon, which was derived from agricultural crops, the cost of turning one agricultural commodity into another (i.e., turning sugar into oil) was never going to be economically feasible as the cost of sugar is never more than about a quarter of the price of most commodity plant oils, such as corn oil, soybean oil, and rapeseed (Canola) oil. Moreover, it is not a question of turning one ton of sugar into one ton of oil. Microorganisms are not that efficient; it takes about 5 tons of sugar to make 1 ton of oil (see Ratledge & Wynn, 2002). Therefore either some zero-cost carbon source had to be found or oils had to be identified that exceeded the prices of the usual commodity oils by a considerable margin in order for SCOs to become economically viable propositions. There was no value in developing SCO processes that simply produced facsimiles of existing commodity plant oils.

In spite of these obvious economic limitations, considerable work on the production of microbial oils took place from the 1920s through to the late 1950s. This work laid the foundation for understanding lipid production in microorganisms. In brief, it was established that:

- The number of microorganisms capable of accumulating oil to more than 20% of their biomass weight was relatively small in comparison with the total number of species.
- Oil-accumulating microorganisms were mainly species of yeast and fungi; only a few bacteria produced much extractable edible oil. The oil produced by these

yeasts and fungi was, like plant oils, mainly composed of triacylglycerols having component fatty acids (FA) that were, in almost every case, similar to what had already been recognized in plant oils.

- Some algae were recognized as producing fairly high amounts of lipid, but this lipid tended to be more complex than that produced by yeasts and fungi, as it included the lipids of the photosynthetic apparatus; nevertheless they still contained the same FAs that occurred in plant oils. Some PUFAs were observed to be similar to those found in fish oils.
- Oil accumulation in oleaginous microorganisms could be increased by starving the cells of a supply of nitrogen or a nutrient other than carbon. The cells responded to deprivation of nitrogen in the growth medium by entering into a lipid storage phase in which excess carbon, still present in the medium, was converted preferentially into storage lipid (triacylglycerols) materials. If the cells were subsequently returned to a situation in which the missing nutrient was now made available, the oil reserves could be mobilized and re-channelled into cellular materials. Lipid accumulation was, therefore, a stress-induced response with the oil being an intracellular storage material that could reintroduce carbon and energy into the metabolic processes of the cell when the prevailing conditions were appropriate.

These views were upheld by extensive investigations, involving both biochemical and molecular studies, carried out on the mechanism of lipid accumulation in oleaginous microorganisms (see, for example, Ratledge & Wynn, 2002 for review).

A typical profile for the accumulation of lipid in an oleaginous microorganism is shown in Fig. 1.1. This figure shows that lipid accumulation in a microbial cell begins when nitrogen is exhausted from the medium. The medium, therefore, has to be formulated with a high C:N ratio to ensure that nitrogen is exhausted while other nutrients, including carbon, remain in excess. In practice, the C:N ratio is about 40–50:1, although the optimum ratio needs to be determined for each individual organism. To produce the greatest number of cells, the concentration of nitrogen and carbon may need to be increased while keeping them in the same proportion; this enables the balanced growth phase to continue until it reaches the maximum biomass density that the fermentor can sustain before the lipid accumulation phase begins. In practice, it is probably advantageous to cultivate cells using NH_4OH as a pH titrant in the fermentor so that cells can grow at their fastest rate without any nutrient limitation and then, when cell density is at its highest optimum level, switching to NaOH as a titrant, while ensuring that the supply of carbon is still in excess. This switch in titrant results in immediate N exhaustion in the culture medium so that lipid accumulation commences when the greatest possible biomass has been achieved (see Ganuza et al., 2008). There are occasionally suggestions that lipid accumulation may not require exhaustion of nitrogen from the growth medium and that the process of oleaginicity is growth-associated in some microorganisms—see, for example, Eroshin et al., 2000, 2002. In other words, lipid accumulates in the cells as they continue to multiply.

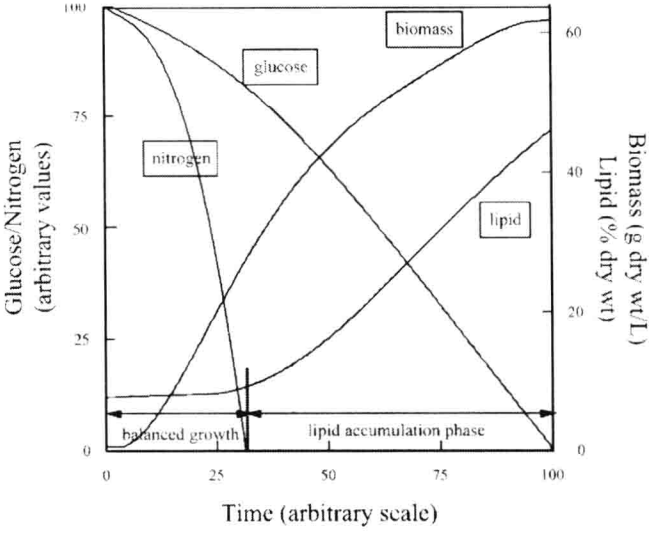

Fig. 1.1. Idealized representation of the process of lipid accumulation in an oleaginous microorganism. The composition of the culture medium is formulated so that the supply of nitrogen, which is usually an ammonium salt, is growth limiting. After its exhaustion, cells do not multiply any further, but they continue to assimilate glucose (the usual carbon feedstock). This glucose is then channeled into the synthesis of storage lipid (triacylglycerol) within the cells. The extent of lipid accumulation depends upon the individual microorganism—lipid contents may vary between 20 and 70% of the biomass.

However, when the growth patterns of these organisms are closely examined, it is apparent that these organisms, which are, in fact, very few, are slow-growers. They clearly have some metabolic impairment. In such cells, glucose is being assimilated into the cells faster than it can be converted into cell biomass and, consequently, its excess is then diverted into the synthesis of a storage material, such as a lipid.

Although attempts were made in Germany during World War II to produce microbial fat to supplement the meager supplies that could be obtained from conventional sources (mainly animal fat with a little plant oil), these efforts were limited. However, some oil-rich biomass production was achieved with fungi. The fungi, mainly *Oidium lactis* (now *Geotrichum candidum*), were grown on waste lactose (from a cheese creamery) or agricultural waste materials (Bunker, 1945, 1946, 1963; Ledingham et al., 1945), and the resultant cells seem to have been fed mainly to army horses after being molded into bricks using hay and straw (Bunker, 1946). Some biomass may have been included in soups and sausages for human consumption, but they were mainly viewed as a protein supplement rather than a source of fat. Although feeding the oil-rich fungi to army horses may sound rather trivial, the German army during this period had approximately one million horses to support and, using unconventional sources of feed material, was clearly considered reasonable. There does not, however, seem to have been any extraction of the oils and, even with

the amount of effort that had gone into developing these processes, the arrival of the first SCO was still some way off.

The development of efficient large-scale production of microbial oils (and, indeed, all microbial products) was limited by the availability of appropriate large-scale fermentors necessary to produce the biomass (microbial cells) at high densities (over 50 g dry wt/L). Laboratory-scale fermentors were relatively unheard of until the 1950s, and industrial-scale stirred tank fermentors were rare. We see this lack of technology evidenced by the UK's need to transfer the technology for penicillin production in the early 1940s (which had used static cultivation of *Penicillium chyrsogenum* in adapted hospital bed-pans) to the US, which had the only accessible stirred tank bioreactors in the world. This technological deficit was a clear limitation not only to microbial oil production but also to almost all other microbial products that needed aerated, submerged cultivation systems. Some fermentors existed in many countries to produce beer and related materials, but these were for the anaerobic production of microbial products and had no facilities for aeration or stirring. Moreover, most were open vessels and, therefore, were prone to airborne contamination, which would have been disastrous for any process seeking to produce food-grade materials.

The major stimulus for developing large-scale fermentation technology and, from it, the production of laboratory-scale fermentor units that would enable the necessary experimental work to be carried out, was probably the advent of single cell protein (SCP) production in the late 1950s. Several petroleum companies—principally BP Ltd. of the UK—began to explore the conversion of *n*-alkanes, unwanted waste materials from the initial phase of fractionating petroleum oil, into edible biomass. Yeasts (especially *Yarrowia lipolytica*—formerly known as *Candida lipolytica*) were found that could grow rapidly on the alkanes, but stirred and aerated fermentors were essential to achieve optimal conversion. The ensuing biomass was rich in protein (about 50% w/w) and proved to be a useful major feed material for animals. As the manufacturers felt a little uneasy about describing their product as "microbial protein," the name SCP was coined as an appropriate euphemism to disguise the origins of the material.

This period of SCP production ended because of unfavorable economics in 1975 with the price escalation of crude petroleum oil and the maintenance of the low price of soybean meal—the major competitor of the SCP—in the USA. But, at the end of this period, the world had developed systems for submerged microbial cultivation to an unparalleled degree. Biotechnology had arrived, and not just for SCP production. Production of antibiotics, amino acids, and organic acids, such as citric acid, now used sophisticated, stirred tank fermentation technology which had replaced the cultivation of microorganisms in static cultures that primarily used shallow tray systems.

With new technology becoming widely available (not forgetting that the availability of laboratory-scale fermentors at a reasonable cost allowed research to be carried out at the 1–2 L level), interest in producing microbial oils once more re-emerged in the mid-1960s (Kessell, 1968; Ratledge, 1968). However, enthusiasm for producing such products had largely waned since plant seed oils were now inexpensive and

there was no prospect of producing oils from microbial sources that could rival their price. There were, though, some prospects of producing one or two "rare" microbial oils (Shaw 1965,1966a, 1966b) that were not readily available from conventional plant sources, but these ideas were still embryonic and lacked focus because the market for such materials was very uncertain. The examination of microorganisms carried out by Shaw (1966b) focused on identifying possible sources of arachidonic acid (ARA; 20:4n-6)—not for use in human nutrition (for which nothing was known at that point), but as a chicken-flavor material! Only after this work had been done did he realize that chicken flavor was not due to ARA, but instead to some entirely unrelated compound. The work of Shaw (1965,1966a), however, proved invaluable for identifying microorganisms that might be used for the production of various long chain PUFAs.

The other main development that occurred in the early 1960s, which was of considerable importance for the study of microbial oils, was the development of gas chromatography. Previously, FA analysis had been laborious and tedious. It also required relatively large amounts of material. Gas chromatography altered all this; one could very quickly analyze a number of oils and fats for their component FA and use just milligram amounts of material. The stage was, therefore, set for a reexamination of microorganisms as potential sources of oils and fats; this shift can be seen in the seminal work of Bob Shaw, mentioned above.

Developments in the Last Quarter of the 20th Century

Work in the author's laboratory (Gill et al., 1977; Botham & Ratledge, 1979; Boulton & Ratledge, 1983; Evans & Ratledge, 1985) consolidated the mechanism of oil accumulation in yeasts being grown in laboratory fermentors, using both batch and continuous fermentations. This work also established that the approximate conversion efficiency of the starting substrate (glucose) to the product (triacylglycerol oil) was maximally 22% (w/w) under optimized growth conditions. But, in spite of understanding a great deal about the process of lipid accumulation,,there was no clear target for which the microbial oils could be considered for commercial development. It was then brought to the author's attention that there might be a small niche market for an oil rich in γ-linolenic acid (GLA, 18:3n-6).

A Process for GLA Production

In the mid-1970s, GLA was only available as a minor component (about 9% of the total FA) in the oil of evening primrose (*Oenothera biennsis*), but this oil was considered, by virtue of its GLA content, efficacious to relieve many symptoms , even for the treatment of multiple sclerosis—a claim that has since been discounted. At the time, evening primrose oil commanded a price of about $50 per kg when most commodity plant seed oils were fetching less than a hundredth of this price. The prospects of a commercially viable SCO were instantly evident since the work of Shaw (1965,