Microalgae Culture Maintenance for Greenhouse Gas Abatement and other Applications

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Microalgae mass cultures have been developed for practical applications in wastewater treatment and specialty foods and feeds production. The "International Network on Microalgae Biofixation of CO_2 and Greenhouse Gas Abatement with Microalgae", with a membership of private and public organizations, is developing microalgae technologies that combine wastewater treatment with renewable fuels production as well as for higher value chemicals and biofuels. The most important issue in developing such large-scale microalgae technologies is the ability to mass culture microalgae strains genetically selected for high productivity and other desired attributes. These strains must be able to dominate under the variable and often extreme conditions present in outdoor open ponds or photobioreactors. This requires large volumes of inoculum, which would be produced in a succession of culture stages, from small-scale laboratory cultures to outdoor photobioreactors and finally large open pond cultures. An original cloned cell would be propagated through several stages, about sixty generations over two to three months, from the initial shake flask culture. During this culture propagation, as well as during the initial isolation, subsequent genetic improvement, strain maintenance and preservation stages, spontaneous undesirable mutations will inevitably arise that could rapidly dominate such cultures. This requires compressing the overall process, from initial natural clone isolation to final large-scale cultivation, into the minimum possible number of generations. This should be a guiding principle for microalgal culture collections.

Introduction

In traditional industrial microbiology, clones of microorganisms are typically isolated from the natural environment, are then mutagenized and clones selected based on desired traits (e.g. overproduction of a particular metabolite). After a successive round of such steps, the improved strains are cultivated in sterilizeable fermenters, thus avoiding contamination. Modern biotechnology differs mainly in the genetic improvement stage of the process, which uses the tools of recombinant DNA and, often, transfers desired characteristics into "platform" microbes (e.g. *E. coli*). Traditional fermentations, such as alcohol and cheese production, are carried out in "open" fermenters, subject to invasions by contaminating microorganisms. In these processes contamination is reduced by combining selective conditions (e.g. low pH), large inoculum sizes and short cycle times (batch fermentations), with maximum possible avoidance of contamination by cleaning the equipment between use, heating the feed, filtering the air supply, etc.

Microalgae mass cultures in open ponds or even closed photobioreactors are similar to traditional fermentations, in that sterilization is not possible (except at relatively small scales, at most a few square meters), and the desired organisms is maintained dominant by a similar combination of strategies. With microalgae cultures, however, the problems of contamination by invading strains of competing algae are exacerbated by the highly variable conditions in the cultivation process, due to variations in daily light intensity, temperature, weather conditions, the long cycle times required for production, and the difficulties of providing an initial clean environment and reducing contamination during culture operations.

For this reason, the two most successful microalgal production systems, in terms of total production, are for the mass culture of *Spirulina (Arthrospira) platensis* (Belay, 1997) and *Dunaliella salina,* which avoid, or at least minimize, contamination by the highly selective growth media they employ, respectively high alkalinity and salinity. In these cases only infrequent or even no re-inoculation is required, with cultures and strains, once established, being maintained in open ponds for long periods, even indefinitely. *Chlorella sp.* and *Haematococcus pluvialis*, the other two algal species cultivated at large-scales, are grown in freshwater media that provides no selective advantage, with maintenance of unialgal cultures achieved by producing large amounts of inoculum and using relatively short cultivation cycles. This makes inoculum production the major factor in the economics of these processes. Some microalgae are cultured on seawater for aquaculture feeds, mainly diatoms (e.g. *Chaetoceros, Cyclotella,*) and flagellates (e.g. *Isochrysis* sp.), and these are also limited by the problems of culture stability and the inability to easily scale-up such processes. In wastewater treatment no attempt is made to culture selected algal strains, limiting the ability to control such process, harvest of the algal biomass, or achieve maximum waste treatment effectiveness. In addition to invasion by competing microalgal species, algal cultures are also subject to invasions by zooplankton grazers, bacterial and fungal parasites, viruses, etc. Their control requires additional techniques, but here the emphasis is on the competition between selected strains and invading "wild" algae.

Here we discuss the issues related to the isolation, development and most importantly in the present context, long-term maintenance of selected algal strains suitable for large-scale algal mass cultures. Such production systems, typically open ponds but also closed photobioreactors, do not provide a significant barrier to invasion by other species or a selective growth environment. Such production systems are also subject to the diurnal and other fluctuations typical of natural environments. It will be necessary to develop standardized procedures for isolation, improvement, maintenance and production of desired strains that can be used in large-scale algal production for wastewater treatment, biofuels and greenhouse gas abatement

Formulation of the problem

A major problem for any microbial culture collection is the selection and dominance during continuous propagation of fast growing clones and random genetic drift. These can be prevented rather readily by using non-growth storage conditions, cryo- or dry preservation (the latter in particular for spores, cysts, and akinetes). Protocols for cryopreservation and storage have been developed for many algae (Day et al., 1995), even rather fastidious ones such as the wall-less saline *Dunaliella* (Levy and Zamir 1994). However, every algal species has different requirements for long-term preservation, and no universal method currently exists. Issues such as recovery efficiency are critical, as is avoiding selection for mutations resistant to the preservation method.

Finding the correct conditions for long-term preservation and high survival recovery for a specific strain is time consuming and not universally successful. For this reason, currently most species in culture collections are continuously propagated through serial transfer under conditions of slow growth (lowered temperature and low light intensities). Indeed, many microalgal cultures used in research have been propagated in such ways for several decades. Even with only two transfers per year, and about a 100-fold increase in cell numbers per transfer (fairly typical values), over a ten year period this would amount to almost 140 generations (doublings, e.g. mitotic divisions), assuming that all cells divide equally. In reality a recovered viable cell would likely have undergone several hundred divisions during this time period in culture.

It is difficult to predict or, until recently, measure, how much genetic variability and, even more important, selectivity would be introduced by such a culture maintenance regime, but it is clearly a significant problem. For example, during several studies of strains obtained from culture collections as well as freshly isolated strains, the cultures exhibited repeated and marked variations in performance (e.g. maximum growth rates, productivity), which can be attributed only to genetic selection during the course of sub-culturing during the research (Weissman, unpublished). Such genetic shifts have been also been observed in the trichome orientation of some clonal and axenic strains of *Arthrospira* after repeated subculturing for one year (Muhling et al., 2003). Such selection of novel strain characteristics in continuous cultures or successive transfers is well known and requires no further discussion.

Recent developments in the use of molecular techniques to decipher genotypic relationships may provide a tool for the detection and selection of species and strains as has recently been done for Spirulina (Arthrospira) strains collected from culture collections and natural habitats (Scheldeman et al., 1999). However, as discussed below, cryopreservation, or other non- or slow-growing maintenance techniques for the cultures, is only one issue in the maintenance of desirable, at present difficult to define, characteristics of selected algal strains required for algal mass cultures.

Selecting algal cultures and strains for mass culture

Another, related, problem is that algal strains that originally possessed the ability to mate and undergo a sexual cycle might loose this ability due to random mutations if cells are not allowed to undergo the sexual cycle after several mitotic generations. More generally, most cultures in collections stem from single cells, representing only one clone out of an entire population. It is well known that natural assemblages of microbes, including microalgae, contain a great deal of genetic diversity. Clonal cultures are desired where genetic improvements are the objective, strain diversity where the objective is to provide a genetic resource base from which to select for the most favorable characteristics.

In the case of microalgae for large-scale mass cultures, both types, wild-type and genetically improved strains, would be desired, at different stages of the development process and for different applications. At present, the low-cost open pond mass culture of microalgae is restricted to only a handful of species and it remains a major challenge to cultivate most algal species outside of the laboratory (and even there success is far from universal). The key issue is the relative growth rate of the selected species *vs*. that of potential contaminants under the operating open pond or photobioreactor conditions. If the selected species or strain grows slower than a competing invading species, it will eventually be displaced. This requires frequent start-ups large-scale inoculum production. To minimize this problem, the strain should be genetically as close to the original wild-type as possible, and the wild-type should be selected by its ability to grow and compete well in the production system.

One approach to this objective has been to use the scaled-down versions of the production ponds or closed photobioreactors as selection ("enrichment") devices, with the dominant algal strains being the ones selected by the local environmental conditions (e.g. water source, temperature, insolation, etc.) and imposed operating conditions (dilution rate, pH, etc.). In such selection processes more than one species would dominate, depending on the bioreactor operating conditions and, possibly, stochastic effects. From among these enriched cultures, additional characteristics could be selected for, in particular their ability to be easily harvested by settling (e.g. "bioflocculation"), a major bottleneck in low cost algal biomass production.

These self-selected, dominant cultures would be expected to contain significant genetic variability, as the pond or photobioreactor environment is far from uniform. Such selected, non-clonal, cultures could then be used in algal mass cultivation, in particular for wastewater treatment where the primary concern is the culture productivity, which directly translates into O_2 production for waste oxidation and sequestration of waste nutrients, as well as ease of harvesting and culture stability. Maintaining genetic variability in self-selected algal cultures used in such applications would be one goal of the culture preservation process. However, at present cultivation of specific species in wastewater treatment ponds has not yet been attempted and requires considerable research and development.

Genetically improving algal strains for mass culture

More generally, it is desirable and even necessary to select for mass culture algal strains with specific attributes, such accumulation of secondary or even primary metabolites (e.g. pigments, oils, carbohydrates, etc.). In these cases it is necessary to generate desirable strains from the enrichment cultures through further selection and/or mutagenesis or by more advanced genetic manipulations. Alternatively, it would be possible to obtain from culture collections strains that already exhibit the desired characteristics, at least in part, and then further improve these. In either case, it may be of interest to also enhance other characteristics of these organisms, such as improved light conversion efficiencies, which would directly reflect on achievable productivity (see below). At

present, the latter approach is the one generally followed, although the danger is that, because of prolonged maintenance in laboratory culture collections, their growth characteristics may no longer be suitable for production in open ponds or even closed photobioreactors. However, use of enrichment cultures is often hampered by the difficulties of growing and manipulating such cultures in the laboratory.

In any case, genetic systems for traditional "breeding" of any of the currently commercially used algal strains are lacking. Therefore, other methods have to be used to generate new strains with specific traits. Although methods and strategies for successful nuclear and organelle transformation have been reported for several microalgae (for examples: Leon-Banares et al., 2004; Zaslavskaia et al., 2001, Geng et al. 2003), these methods are still very laborious and fully developed systems for genetic engineering of microalgae are generally lacking, except for some cyanobacterial strains. Another complication is that consumer and environmental concerns sometimes limit the application of such advanced technologies.

Approaches to manage the strain stability problem

In the above discussion two main approaches to selection of algal strains for large-scale cultivation were discussed: use of a scaled-down open ponds or photobioreactors for selection of competitive strains, or reliance on culture collection strains with known desired attributes, such as high contents of carotenoids or other pigments, fatty acids, etc. It may be possible to subject the culture collection strains to a selection step in a scaled-down culture system, but this would likely be unsuccessful as culture collection strains would have limited genetic diversity. In any event, this approach has not yet been reported. This again highlights the need to consider the suitability for algal mass culture of culture collection strains as they have been up to now maintained and managed. And, most importantly, it also emphasizes the need to reduce to an absolute minimum the time and generations between strain isolation and their applications to mass cultures.

To reduce the generations between isolation, screening, genetic selection/manipulation for improved strains to an absolute minimum, several techniques can be considered. Micromanipulation techniques could be employed in the initial isolation of the strains. Automated micromanipulators allow the isolation of single cells directly from water samples or from plates, drastically reducing the number of doublings during the initial isolation. Note that the number of clones generated per species should be as large as possible. After a minimal initial growth, the cloned cultures would be cryopreserved to ensure maintenance and stability of the initial genetic variability.

The next step would be strain improvement, which can use the classical mutagenesis-selection techniques or DNA insertional mutagenesis, or include transfer of exogenous DNA. At present the only well developed method to perform microalgal strain improvement is classical mutagenesis, using chemicals, UV or radiation. These would be followed by selection (positive or negative) from among the surviving cells those with desirable characteristics. For example, to improve solar energy conversion efficiencies, mutations with reduced antenna pigments are being investigated (Nakajima et al., 2001; Polle et al., 2001, 2002). However, such mutants would, although more productive be somewhat paradoxically less competitive that the wild-type strains. Thus it is imperative that such strains not be further weakened by additional mutations, such as often occur when the techniques of classical chemical or UV mutagenesis are used. Unfortunately, it is not easy to detect how many mutations per cell were introduced through application of classical chemical or UV mutagenesis. Therefore, mutants with desirable phenotype, such as high solar conversion efficiencies, should be backcrossed to the wild type several times to eliminate any other mutations negatively impacting their fitness.

Again, for such strain improvement programs, involving a combination of selection and screening techniques, it is imperative to minimize the number of generations. The technique of cell sorting could be applied for identification of single cells with desirable characteristics, greatly reducing the number of doubling times necessary compared to other screening procedures. After identification of mutant strains with the desired qualities they should be brought back into outdoor cultures as soon as possible. Of course, samples should be cryopreserved as part of this process.

Strategies for microalgal inoculum production

As discussed in the introduction, microalgae can be mass cultured if a selective environment allows the exclusion of all or most other potential competitors, as is the case in the commercial production of *Spirulina* (cultivated at high alkalinity) and *Dunaliella* (high salinity). Otherwise a large amount of inoculum must be used for the production process (Sarada et al., 2002). This requires algal strains that grow fast under conditions of the inoculum generation stage while being also highly productive in the production system (Huesemann et al., 2003). This is a major issue in any large-scale algal production process, where the cost of the inoculum production could easily dominate the overall economics, as it does with *Chlorella* and *Haematococcus* production.

Inoculum production requires a succession of stages that rapidly amplify the initial small laboratory culture to hundreds and then thousands of square meters. Emphasis is on speed, to avoid the culture being overtaken by fast growing contaminants. For any application involving large-scale production of algae for waste treatment, biofuels production and greenhouse gas abatement, the final stage of this process would be a large outdoor mass culture ponds.

A conceptual low-cost inoculum production process (Table 1) was proposed (see Benemann, 2003) in which the culture is increased in scale over ten successive stages, from a small initial laboratory culture to inoculate a 400 hectare pond system. The overall cost of the inoculum process increases the capital cost of the culture system by about a third. Each stage increases the algal culture by ten-fold, or one billion-fold overall, starting from the laboratory culture (1 g dry weight) to the standing biomass in the ponds (1,000 tons dry weight), about 30 generations. The starter laboratory culture, if grown from a cryogenic sample of 1000 cells, would also require a billion-fold expansion, or 30 generations (assuming 100 pg/cell). Although this example is only illustrative, it demonstrates that inoculum production needs to be carefully managed, to avoid the genetic selection and drift that could otherwise easily result.

Growth Stage (from lab to pond)	Area m^2	Unit Cost $/m^2$	$,000 /stage
1. Initial Flask	<0.01 m^2	n.i.	n.i.
2. Bottles Culture	<0.1 m^2	>5,000	5
3. Lab Cultivation	0.4 m^2	5,000	2
4. Sterilizeable bioreactors	4 m^2	2,000	8
5. Small Photobioreactors	40 m^2	300	12
6. Large Tubular Reactors	400 m^2	200	80
7. Plastic Sleeve Reactors	0.4 ha	50	200
8. Covered Ponds	4 ha	20	800
9. Open Lined Ponds	40 ha	6.5	2,600
10. Production Ponds	400 ha	2.5*	10,000
TOTAL SYSTEM	445 ha	3.4*	13,700

Table 1. Proposed Mass Culture Inoculum Production Process for Large-scale Microalgal Mass Cultures. Note: "n.i." not included, minor costs. *Costs only for unlined large-scale open ponds; harvesting, processing, infrastructure or other capital costs not included.

Microalgae culture collections and genetic resources

The problem of genetic drift in connection with maintenance of cultures has been recognized for several decades and led to efforts to cryopreserve algal species (Ben-Amotz and Gilboa, 1980; Flassch et al., 1975; Morris 1976). However, only in recent years has genetic drift been recognized as a significant problem in microalgae biotechnology. A European initiative "*The Conservation of a Vital European Scientific and Biotechnological, Resource: Microalgae and Cyanobacteria (COBRA; http://www.cobra. ac.uk/)*" was recently founded to help preserve biodiversity and ensure genetic stability of the culture collections, by, among others, developing cryopreservation techniques for currently not or poorly preservable organisms. Culture collections need to use such approaches to improve the success of algal biotechnology.

A new culture collection for species of the genus *Dunaliella* was set up recently at Brooklyn College of the City University of New York (DCCBC) to provide a variety of species and strains for education, research and commercial development. DCCBC obtains water samples from natural habitats and isolates multiple clones from each, to help preserve at least a modicum of the genetic plasticity present in the natural environment. Moreover, it is essential for heterothallic species of *Dunaliella* (Lerche 1937) to maintain multiple clones of one species, because at least two clones of opposite mating types are needed to undergo the sexual cycle. At DCCBC algal strains are currently maintained on agarose plates, but to circumvent genetic drift long-term storage methods are under investigation. Initial experiments showed that cysts or zygotes of the species *D. viridis* and *D. tertiolecta* can be stored for more than 12 months either in a dry state or in liquid medium deprived of nutrients (unpublished). Cryopreservation of strains according to Levy and Zamir (1994 will be used in the future for alternative long-term storage.

Discussion

As outlined above, both genetic plasticity and stability during microalgae culture maintenance is essential for biotechnological applications of microalgae. Therefore, collection and preservation of environmental samples with natural assemblages in reasonable quantities should be considered. In addition, clones of algal cells must be preserved such as to avoid genetic selection and drift in culture collections. Although the issue of long-term storage of cultures has recently started to be addressed by several major algal culture collections, these problems deserve greater attention and scrutiny, in particular in the context of the strain selection, genetic improvements, inoculum production and mass culture issues discussed above.

Recently the "International Network on Biofixation of CO_2 and Greenhouse Gas Abatement with Microalgae" was established under the International Energy Agency Greenhouse Gas R&D Programme (www.ieagreen.org.uk), with several large energy companies and research agencies joining this Network (Pedroni et al., 2003). The objective of the Network, and of its member organizations, is to advance practical R&D in this field. A technology roadmap (Benemann, 2003) outlines the basic approach to this goal, which is to develop algal strains with high productivity (>100 metric tons biomass per hectare per year), and to then mass culture such strains in open ponds for production of renewable fuels, wastewater treatment, fertilizer production, and related applications, singly or in combination. Critical to the achievement of this goal will be the development of techniques for the collection, enrichment, selection, isolation, identification and preservation of desirable algal strains, directly from the natural environment and after needed genetic improvements. The international collaborative R&D efforts being carried out by the members of the International Network require the participation of researchers in many areas, but most particularly those dealing with algal culture collections, the foundation of any practical applications.

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