THE UNIVERSITY of EDINBURGH

Edinburgh Research Explorer

Algae for biofuel

Citation for published version:
Collins, S & Bull, J 2012, 'Algae for biofuel: will the evolution of weeds limit the enterprise?' Evolution, vol 66, no. 9, pp. 2983–2987., 10.1111/j.1558-5646.2012.01652.x

Digital Object Identifier (DOI):
10.1111/j.1558-5646.2012.01652.x

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Author final version (often known as postprint)

Published In:
Evolution

Publisher Rights Statement:
Free in PMC.

OPEN ACCESS

Algae for biofuel: will the evolution of weeds limit the enterprise?

J. J. Bull and
Section of Integrative Biology, Institute for Cellular and Molecular Biology, Center for Computational Biology and Bioinformatics, University of Texas, Austin, Texas 78712, bull@utexas.edu

Sinéad Collins
Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh, The King's Buildings, West Mains Road, Edinburgh, EH9 3JT United Kingdom, s.collins@ed.ac.uk

Abstract

Algae hold promise as a source of biofuel. Yet the manner in which algae are most efficiently propagated and harvested is different from that used in traditional agriculture. In theory, algae can be grown in continuous culture and harvested frequently to maintain high yields with a short turnaround time. However, the maintenance of the population in a state of continuous growth will likely impose selection for fast growth, possibly opposing the maintenance of lipid stores desiriable for fuel. Any harvesting that removes a subset of the population and leaves the survivors to establish the next generation may quickly select traits that escape harvesting. An understanding of these problems should help identify methods for retarding the evolution and enhancing biofuel production.

Despite old and reasonably accurate forecasts that the world would experience 'peak' oil production near 2015, serious public and political attention to alternative energy has only recently gained momentum. Biofuels hold promise as one of the few sustainable sources of liquid fuel, and indeed, ethanol from land plants has been available for some time in several countries (Gray et al. 2006). However, the net energy gain from land plants is not impressive, and fuel crops compete with food crops, forcing governments into difficult choices.

A much-touted source of future biofuels is algae, and companies on both sides of the Atlantic are attempting to develop algal fuels (Chisti and Yan 2011). Algae offer several advantages over land plants as a source of fuel: competition with food crops is much reduced with algae, and the projected net energy yields from algae vastly exceed those from land plants (Chisti, 2008). To date, however, the economical conversion of algae into fuels faces many technical challenges (Davis et al. 2011), and large-scale production, or even sustained production on small scales, has not been put to the test.

The point of this article is to highlight some likely evolutionary challenges that will confront the easy use of algae for biofuels. A seeming advantage of algae is that they can be grown and harvested in a fundamentally different manner than is usual for agricultural crops – algae can be grown as a continuously reproducing population and harvested to maintain high yield, much as a managed, wild foodstock of fish or game. Yet this propagation and harvesting will subject populations to selection, and in many cases this selection should be antithetical to maintaining algae suited for biofuel production. An understanding of these evolutionary principles will be needed to design growth and harvesting methods that reduce the negative impact of selection. There will also be profound ecological problems unique to algae in open ponds, but we do not address those here.

The standard agricultural approach applied to algae: discrete culture

Modern agriculture has been immensely successful in selecting a plethora of land plants that produce large quantities of edible biomass. When wild ancestors of a domestic species are known, the wild strains nearly always produce less of the edible portions than do the domestic strain. In some cases the genetic bases of the domestication are known (corn vs. teosinte) (Doebley 2004). The desired characteristics of the domesticated species would not be adaptive in the wild, but humans have subverted natural selection and given a reproductive advantage to plants with the desired traits, and even now we continue to create better and better domestications. This artificial selection has been applied by propagating descendents (seeds, cuttings) of the individual plants with desired traits, while avoiding the propagation of undesired individuals. The desirable traits are maintained either because the crops are continually selected for individuals with the desired traits or because seed stocks of the good strains are used for planting each season. Although we can use the same general methods to create algal strains good for biofuels, the most efficient methods of algal propagation will work against maintaining strains with those characteristics suitable for biofuel (Table 1).

To apply the discrete culture protocol in the growth and harvesting of algae, an algal-free vat or pond would be inoculated with small numbers of a strain bred or engineered for fuel. Assuming that the strain was a microalga reproducing by fission and the pond is at least an acre-foot, a starting culture of 10^4 cells would need to be grown for ~ 40 generations (an increase of 10^{12}) to attain an abundance sufficient for harvesting. The reservoir would then be completely harvested– cleared of algae – and the process repeated. Drawbacks of this approach include the 40 generations required to reach harvestable density during each cycle and the cost of eliminating nearly all algae from the water. Failure to eliminate all algae before the next cycle would select weedy strains (see below).

An 'inoculum train' alleviates some of these problems because of the exponential growth of cultures. If the volume of the harvested reservoir is V and a small inoculum requires 40 generations to reach harvestable density, an intermediate-sized reservoir of 0.01V can be inoculated and grown for 33 generations, then dumped into the large reservoir for the final 7 generations before harvest. With 5 intermediate reservoirs whose inoculations are staggered by 7 generations each, the large reservoir can be harvested 'continuously' every 7 generations instead of every 40, and the total excess volume needed is only 0.05V. The principle can be extended to an arbitrary number of intermediate-sized reservoirs.

The alternative of continuous culture

The ability of algae to reproduce continuously allows a different approach to be used in their culture and harvesting. As in the above scenario, a reservoir is inoculated and the culture grows to high density. Now, however, instead of harvesting the entire population, only half is harvested. The residual, unharvested population is merely one generation from attaining harvestable density again. Thus, in the course of 40 generations following the first harvest, the continuous culture protocol recovers 20 times the harvestable population size of algae, whereas the discrete method recovers a tenth as much. If time is a major limiting factor in the economics of fuel production, this latter method offers a major improvement over the discrete method. Use of an inoculum train with the discrete culture reduces the discrepancy between the two methods but requires multiple transfers of cultures and some extra infrastructure. (Note that we use 'half' as an example; analogous arguments can be made for harvesting other fractions.)

The sustained, harvesting of half the population is good for maintaining high yields in the short run, but it also imposes selection. All else equal, selection here strongly favors fast

algal growth: the population is being maintained in a constant state of growth, and faster growers will prevail over slower growers (e.g., Mikkola and Kurland 1992). Since algal populations in bioreactors are likely to be large, with a constant supply of beneficial mutations, we expect them to respond quickly to selection and evolve to grow more rapidly.

The impact of selection for fast growth

Evolution of fast growth is likely to be counterproductive to biofuel production. The basis of this suggestion is that not all algal biomass is equally good for biofuels; lipids are particularly desired by industry, but the basic cellular scaffold of an algal cell is not. Thus it is not enough to merely grow algae, they need to be filled with a particular form of stored carbon. Yet any metabolic process that uses energy and other cellular resources to produce stores necessarily diverts resources from cell growth (Dragone et al. 2011). Stores, usually in the form of starch or lipids, are no doubt beneficial in a natural environment where periodic resource limitation is a fact of life, but not in a pond continually favorable to growth. It is thus expected that mutants abandoning stored reserves in favor of reproduction will arise and be favored, eventually displacing their ancestors that still maintain lipid reserves. Oddly enough, we could find no experiments directly testing whether or not there is a tradeoff between rapid growth and carbon storage (starch or lipid) in microbes during long-term cultures. While the existence of a tradeoff between growth rate and yield is also possible, there is only limited evidence for such a tradeoff in large microbial populations (Velicer 1999, Novak et al. 2006).

To date, particular microalgae have been put forward as candidates for biofuel production based on their existing phenotypes (Rasoul-Amini et al. 2011). Screening protocols have been used to find natural isolates that have high lipid stores (Abou-Shanab et al. 2011) and to find isolates that increase these stores substantially under set culture conditions, usually changes in nutrient levels. Studies evaluating various microalgae for use as biofuels focus on those that increase their production of lipids under short-term nutrient limitation (usually nitrogen limitation; Amaro et al. 2011), where the stoichiometry of nutrients limits the production of nitrogen-rich proteins so that cells must somehow deal with a relative excess of carbon in order to make new cells – thus putting excess carbon into lipids.

Unless there is a fundamental evolutionary constraint linking the production of lipid stores to faster growth, is is likely that lineages will explore other possibilities for increasing growth rate under low-nitrogen (or other low-nutrient) conditions. For example, most microalgae probably secrete or leak carbon in some form (Vilchez et al. 1997), and it may be more beneficial to come up with a way to secrete excess carbon rather than invest energy in making lipids. Since there is variation in how microalgae react to nitrogen limitation (Amaro et al. 2011), and the supply of beneficial mutations is high in large populations, it is plausible that no evolutionary constraint exists that would maintain the correlation between storing lipids and making new cells under N limitation.

One way to avoid or slow the evolutionary deterioration of lipid accumulation is to modify algae to force maintenance of the correlation between lipid storage and reproduction – for example by disabling other potential pathways for "dumping" excess carbon, such as starch formation or carbon excretion. Low-starch mutants have resulted in increased lipid production in the short term (Wang et al 2009, Li et al. 2010). Long-term selection experiments must now be performed with these modified algae to see whether an evolutionary constraint on responding to selection for rapid growth under N limitation has been introduced, or whether using starchless mutants would merely delay the evolution of decreased lipid storage. The use of starchless mutants may be especially effective if combined with a modified culture method (see point 1 below) that simultaneously selects on

the maintenance of lipid stores alongside fast growth. It should be noted that most studies that look for constraints on evolution in large microbial populations find weak or no constraints on phenotypic adaptation, even though constraints on particular genetic solutions exist (Korona 1996; Novak et al. 2006; Hall et al.2010), so that it is unlikely that a phenotype not under selection (lipid storage) will be maintained in the face of strong selection for another trait (fast growth).

Selection imposed by harvesting

The harvesting method itself will also effect selection, whichever protocol is used. Under continuous propagation, an easy method is required to remove much but not all of the algal population every generation. Consider flocculation, now being entertained as a harvesting method. With flocculation, a chemical is added to the pond, algae clump, sink, and are removed. In turn, the non-flocculated algae remain behind in the pond to reproduce and generate the next population for harvest. As can be anticipated from a century of quantitative genetics applied to animal breeding, this protocol will eventually and perhaps quickly produce a population recalcitrant to flocculation. The same outcome is expected with virtually any harvesting method that allows the non-harvested individuals to breed the next generation. Even the attempt to harvest or otherwise eliminate the entire population of algae before the next introduction is likely to select escape mutants that will become increasingly problematic in future generations. Of course, methods can easily be developed that kill all algae in a restricted environment, but the goals of minimizing cost and maintaining the water suitable for subsequent cultures will be inimical to many of the otherwise easy methods of algal elimination.

The nature of selection in an inoculum train will depend on the manner of transfer – whether algae alone are transferred at each step or the entire body of water is transferred. Algae will be selected to remain behind at transfer, but the long term impact of this selection on culture productivity will be ameliorated by the constant influx of new individuals. The selection imposed by harvesting may interact with selection for fast growth (a point raised by a reviewer). For example, algae that stick to environmental surfaces or are otherwise sequestered in parts of the reservoir with reduced harvesting may sacrifice high growth rate to persist in those micro-environments. It may prove difficult to predict the multivariate phenotype evolution of algae under these conditions, but there is generally no reason to expect the net evolution to be one that maintains high biofuel performance.

Thwarting evolution

The evolutionary problems faced in the attempt to grow and harvest algae for industrial purposes are specific manifestations of the evolution of weeds. Beginning with Harper (1956) and Baker and Stebbins (1965), there is now increasing attention to weeds as an evolutionary problem (Ellstrand et al. 2010). And despite the differences between standard agricultural crop practices of discrete propagation and the continuous propagation method that could be used for industrial algae, there are several parallels between the evolution of weediness in both contexts. In particular, weeds often persist as continuous populations in the agricultural fields. Even though the crops themselves are replaced each season, the weeds in one year are descended from the weeds at that location in the previous years, evolving continuously to avoid harvesting and to reproduce ever more. And in several crops, the weeds themselves are evolved directly from the crops they infest, much as we suggest will result with algae (Ellstrand et al., 2010, Gross and Olsen 2009).

There may be no easy way to fully prevent detrimental algal evolution while also enhancing biofuel recovery. Nonetheless, an awareness of the problems may point toward at least partial solutions. It was noted above that discrete culture limits selection/evolution of weedy

species, at least to the degree that the culture can be completely harvested before the new culture is inoculated. Some ways to limit evolution of weediness in continous culture protocols are evident:

1. The most obvious solution is a blend of discrete and continuous propagation. It may be possible to realize several harvesting cycles under continuous propagation before weeds evolve to a level that impacts yield. The population is then completely expunged and replaced with fresh stock. An inoculum train can be used for the discrete propagation portion of the protocol, further reducing the time between harvests.

2. It may be possible to apply selection that favors maintenance of lipid stores in the algae despite selection for rapid growth. Starvation or exposure to other harsh conditions may kill algae with few reserves, and if such conditions precede growth phases, the harsh conditions may impose enough selection to keep the final cultures from being overwhelmed by algae lacking reserves. While this will ensure that cultures maintain some form of reserves, it would not favour lipid over carbohydrate reserves, but could be effective if combined with selection for, say, a decrease in cell density, which would favour lipid over carbohydrate as a strategy for avoiding starvation. It is possible that any evolution resulting from this selection will decrease yield. An inoculum train provides a simple opportunity to vary selection, with intermediate cultures (in small volumes) subjected to a different type of selection than in other steps and with virtually no loss of time.

3. To reduce selection imposed by harvesting, the harvesting schemes could be cycled, designed to at least occasionally keep the progenitors of future populations, rather than invariably remove algae from progenitor populations. If complementary harvest methods are available, they could be alternately used to retain versus process algae, preventing algal adaptation to escape harvesting. A specific suggestion is to re-add some proportion of the harvested high-lipid, floculating cells to the non-harvested population. For example, it may be feasible to enrich the harvested fraction for high-lipid cells by centrifugation, since high-lipid cells should be less dense than low-lipid cells. If cells are harvested from a well-mixed culture at random, then adding even a small number of high-lipid cells back into the culture will give that lineage a selective advantage and favour high- rather than low-lipid cells (although selection for rapid growth may oppose this advantage for high lipid content). This would not prevent lineages evolving ways to avoid being harvested (such as forming films on walls), but may substantially increase the useful number of harvests between having to re-start cultures from the ancestral population. While this may add some extra work to harvesting, it may be more efficient in the long run than restarting cultures from scratch more often.

Likewise, it may be practical to chose a harvesting method in which the cost of being harvested is outweighed by the advantage of occupying the harvested niche. For example, harvesting from the top of the water column may balance selection to avoid being harvested with the benefit of access to light. Even if it is not possible to fully offset the disadvantage of being harvested, it may be practical to choose a harvesting method that substantially weakens the selective impact of harvesting.

The basic foundations for principles and practices that may be useful in avoiding or directing evolution of algal populations are likely well known to evolutionary biologists. There is also a history of applications of many of these principles in the fisheries industry (Walters and Martell, 2004) and a growing appreciation of the principles in addressing weeds in agriculture (Ellstrand et al. 2010). In these early days of enthusiasm about large scale algal aquaculture, there seems to be little emphasis on evolution. But evolution will

surely happen, and we may hope that evolutionary biologists will find an important role in making this industry a success, and soon.

Acknowledgments

Literature Cited

Abou-Shanab, et al. Characterization and identification of lipid-producing microalgae species isolated from a freshwater lake. Biomass and Bioenergy. 2011; 35:3079–3085.

Amaro HM, Guedes AC, Malcata FX. Advances and perspectives in using microalgae to produce biodiesel. Applied Energy. 2011; 88:3402–3410.

Baker, HG.; Stebbins, GL. The Genetics of Colonizing Species. New York, NY: Academic Press; 1965.

Chisti Y. Biodiesel from microalgae beats bioethanol. Trends Biotechnol. 2008; 26:126–131. [PubMed: 18221809]

Chisti Y, Yan J. Energy from algae: Current status and future trends. Algal biofuels – A status report. Applied Energy. 2011; 88:3277–3279.

Davis R, Aden A, Pienkos PT. Techno-economic analysis of autotrophic microalgae for fuel production. Applied Ecology. 2011; 88:3524–3531.

Doebley J. The genetics of maize evolution. Annu. Rev. Genet. 2004; 38:37–59. [PubMed: 15568971]

Dragone G, et al. Nutrient limitation as a strategy for increasing starch accumulation in microalgae. Applied Energy. 2011; 88:3331–3335.

Ellstrand NC, Heredia SM, Leak-Garcia JA, Heraty JM, Burger JC, Yao L, Nohzadeh-Malakshah S, Ridley CE. Crops gone wild: evolution of weeds and invasives from domesticated ancestors. Evol. App. 2010; 3(5–6):494–504.

Gray KA, et al. Bioethanol. Curr. Opin. Chem. Biol. 2006; 10:141–146. [PubMed: 16522374]

Gross, BL.; Olsen, KM. Evolutionary genomics of weedy rice. ch6. In: Stewart, CN., editor. Weedy and Invasive Plant Genomics. Oxford, UK: Wiley-Blackwell; 2009. p. 83-98.

Hall AR, Griffiths VF, MacLean RC, Colegrave N. Mutational neighbourhood and mutation supply rate constrain adaptation in *Pseudomonas aeruginosa*. Proc. Roy. Soc. B. 2010; 277:643–650.

Harper, JL. Proceedings of the third British weed control conference. UK: The British Crop Protection Council; 1956. The evolution of weeds in relation to resistance to herbicides; p. 179-188.

Korona R. Genetic divergence and fitness convergence under uniform selection in experimental populations of bacteria. Genetics. 1996; 143:637–644. [PubMed: 8725215]

Li Y, Han D, Hu G, Sommerfield M, Hu Q. Inhibition of starch synthesis results in overproduction of lipids in *Chlamydomonas reinhardtii*. Biotech. Bioeng. 2010; 107:258–268.

Mikkola R, Kurland CG. Selection of laboratory wild-type phenotype from natural isolates of Escherichia coli in chemostats. Mol. Bio. Evol. 1992; 9(3):394–402. [PubMed: 1584010]

Novak M, et al. Experimental tests for an evolutionary trade-off between growth rate and yield in E. coli. Am. Nat. 2006; 168:242–251. [PubMed: 16874633]

Rasoul-Amini S, et al. *Chlorella* sp. A new strain with highly saturated fatty acids for biodiesel production in bubble-column photobioreactor. Applied Energy. 2011; 88:3354–3356.

Velicer GJ. Pleiotropic effects of adaptation to a single carbon source for growth on alternative substrates. Appl. Environ. Microbiol. 1999; 65:264–269. [PubMed: 9872788]

Vilchez C, Garbayo I, Lobato MV, Vega JM. Microalgae-mediated chemicals production and wastes removal. Enzyme and Microbial Technology. 1997; 20:562–572.

Walters, CJ.; Martell, SJD. Fisheries ecology and management. Princeton, NJ: Princeton Univ. Press; 2004.

Wang ZT, Ullrich N, Joo S, Waffenschmidt S, Goodenough U. Algal lipid bodies: Stress induction, purification, and biochemical characterization in wild-type and starchless *Chlamydomonas reinhardtii.* Eukar. Cell. 2009; 8:1856–1868.

Table 1

Alternative protocols for growing algae

Method of propagation	Problems
<u>Discrete culture</u> inoculate reservoirs with high-yielding strains, grow, harvest the entire crop. Repeat the inoculation.	Nearly 40 generations needed for culture to reach high density; high cost to achieve complete harvesting; evolution of mutants that escape harvesting. Use of inoculum trains reduces time between successive harvests, but requires extra space and transfer of cultures.
<u>Continuous culture</u> inoculate reservoirs with high-yielding strains, grow, harvest half the culture; one generation later, harvest half the culture again; repeat indefinitely	Selection of weediness – mutants that grow fast and the possible evolutionary decay of biofuel capacity; evolution of mutants that escape harvesting.