GENETIC ENGINEERING OF ALGAL SPECIES

Ann-Sofi Rehnstam-Holm
Clinical Bacteriology, Goteborg University, Goteborg, Sweden

Anna Godhe
Marine Botany, Goteborg University, Goteborg, Sweden

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Summary

Genetic engineering of algae is not common due to problems related to the design of vectors (i.e. plasmids or viruses) that can be successfully incorporated into the algae, accepted by the cell and expressed in a satisfying way. Most studies have therefore been made on the "green yeast" *Clamydomonas reinhartii* and some cyanobacterial species. However, in this review we are presenting examples of studies performed on a broad collection of algal species ranging from cyanobacteria to macroalgae like *Laminaria*. We have included different kinds of applications, within physiology, biochemistry, molecular biology, phylogeny, industry and environmental science. This ongoing and forthcoming research will undoubtedly increase our knowledge and usage of these important and fascinating primary-producing organisms.

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1. Introduction

1.1. What are Algae?

Algae are a heterogeneous group of organisms. They are aquatic or live in damp habitats on land. Some are prokaryotic but most are eukaryotic. Cell size can vary from 1 µm up to tenths of meters and the complexity from a rather simple spherical cell to a highly differentiated plant (Figure 1 and 2).

Figure 1. Ice-floe holding millions of marine diatoms, Weddel Sea, Antarctica. (Photo: Anna Godhe, Marine Botany, Göteborg University, Sweden.).

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Figure 2. Marine kelp, Cape of Good Hope, South Africa. (Photo: Anna Godhe, Marine Botany, Göteborg University, Sweden).

They reproduce sexually, with complex lifecycles, or asexually. Some can produce resting stages called cysts that can survive in sediments for at least 10 to 50 years. The only feature that the algae seem to have in common is their ability to use light to fix carbon from CO_2 and to produce oxygen in the process. However, even this autotrophic mode is not true for all algae. Some have a strict heterotrophic mode of life, while others can switch between obtaining carbon from fixation or by eating other organisms or organic particles. All algae are not related evolutionary, i.e. they do not share a common ancestor, but seem to have evolved on several separate occasions. Indeed, the only really common feature that algae seem to share is the inclination to occupy damp places. The definition "algae" are thus more of a traditional and practical naming and should not be considered as a group of organisms of common ancestry.

1.2. What is Genetic Engineering?

The words "genetic engineering" are also hard to delimit [see also *Methods in Genetical Engineering*; *Genetics and Society*]. We have chosen to interpret it generously here, so that all kinds of genetic work performed on algae will be considered. Strict genetic engineering studies, i.e. the insertion of another organism's gene into the genome of an alga, are scarce, and nearly all work in this field has been performed on very few organisms like the "green yeast" *Chlamydomonas reinhardtii* and some species of cyanobacteria. Thus, in this summary we have included representative molecular studies on genetical diversity, phylogeny and taxonomy as well as physiological mechanisms and applied genetic engineering.

1.3. The Importance of Algae

The use of algae in biotechnological research and industry is significant. Algae play roles as biocatalysts for the production of food, chemicals and fuels and they are becoming important in the development of solar energy technology, biodegradation and bioremediation. In addition, some species of algae are eaten directly by humans. The red macroalgae *Porphyra* sp. is a common ingredient in East Asian cuisine. The markets for other algae, like the microalgae *Spirulina* sp., *Chlorella* sp. and *Dunaliella* sp., are expanding as a food supplement in western world health stores. For instance, *Spirulina* (a cyanobacteria) has a protein content above 70 percent, which also makes it attractive as fodder in the aquaculture industry. Many of these algal species are retailed because of their antioxidant properties.

Algae are also sometimes causing severe problems (Figure3). The expanding international aquaculture industries often encounter severe problems due to harmful algae. Some algal species carry spines that can physically damage fish gills. Other algae produce toxins, which accumulate in filter feeders, like commercially important oysters and mussels. Oysters and mussels are usually not affected, but human consumers might experience different diseases.

2. Classification of Algae

Before one considers bioengineering of algae, it is necessary to define the taxonomic position of these organisms. This is not an easy task since algae are an extremely

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cohesive group of organisms and clearly not relatives in the evolutionary (phylogenetic) sense like animals are. Alga phylogeny can most clearly be visualized as a tree (Figure 4).

Figure 3. Red tide caused by dinoflagellates. Skagerrak, NE Atlantic.
(Photo: Anna Godhe, Marine Botany, Göteborg University, Sweden)

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Many different characters can be evaluated to construct such trees and the most widely used feature today is DNA sequence data (see below; section 5). Such data has provided evidence for the existence of ten major phyla of algae. These are the *Glaucophyta, Euglenophyta, Cryptophyta, Haptophyta, Dinophyta, Heterocontophyta* (including diatoms, brown algae), *Rhodophyta* (red algae), *Chlorophyta* (green algae) and the prokaryotic *Cyanophyta* (cyanobacteria) and *Prochlorophyta*. When more molecular data becomes available, it is highly likely that this division might change. Two other groups of organisms, the apicomplexans and chlorarachniophytes, which contain plastid genomes (the genome of chloroplasts), may in the future be identified as algae. Some groups of algae are closely related to non-photosynthetic organisms (protozoans). One striking example is the relationship between *Trypanosoma*, the cause of sleeping sickness and Chagas disease, and the chlorophyll containing hay infusion organism *Euglena*. Another is the relationship between ciliates (such as *Paramecium*), the apicomplexans (like the malaria parasite *Plasmodium*) and dinoflagellates (like toxic *Alexandrium*). How is this possible? The answer is endosymbiosis, where one or a few endosymbiotic organisms have been incorporated in a host cell, and the movement of genes from one organism to another.

3. Principles of Microalga Culture

To be able to isolate algae from its natural environment one has to mimic both its chemical and physical habitat. The basic problem in establishing algal cultures is the design of the media (see also Algal cell culture). Natural water is very dilute but at the same instance a very complex media. This is why purified offshore water (or artificial seawater) is used as the basis for marine alga culturing media. To this, precisely defined

quantities of major nutrients (i.e. nitrogen, phosphorus, silica), minor nutrients (i.e. copper, zinc, cobalt, manganese, molybdenum, iron, selenium) and vitamins (i.e. B12, thiamin and biotin) are added.

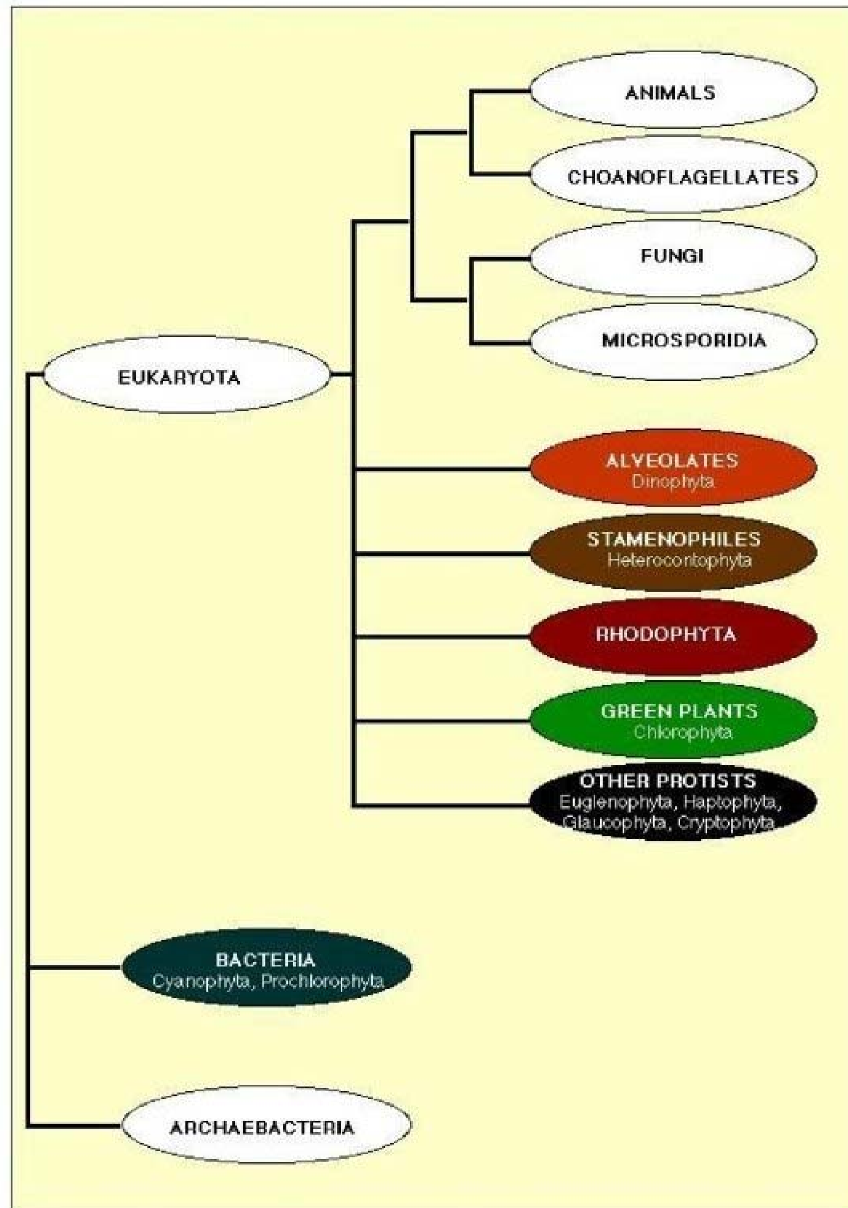

Figure 4. Phylogenetic tree based on ribosomal RNA sequences. Major groups which includes algae are indicated with color (adapted from D.J. Patterson & M.L.Sogin, Tree of Life at http://phylogeny.arizona.edu/tree/phylogeny.html)

Light intensity, light quality and day length are parameters that can have profound effects on algal growth. In general, cultured algae are adapted to rather low light intensities and the temperature range is quite broad.

Beside the physiological parameters, many algae also need specific biological parameters to be able to grow. These parameters are often completely unknown, which

creates problems. Many marine algal species can only be cultivated for some generations in natural, untreated seawater. The exclusion of some of the accompanying species is usually possible (i.e. predators), but often the algae of interest cease to grow after exclusion of all accompanying species (i.e. small flagellates and bacteria). If this is due to the algal species need for prey organisms or if they live in a mutualistic kind of mood, still remains to be solved. Mixed cultures create problems when studying algae. It should therefore be pointed out that, at the moment, there are very limited numbers of algal species that are used in biogenetic engineering studies, since these studies nearly always require one to grow the algal species without other organisms present in the culture (i.e. axenic cultures).

It should also be mentioned here that several species of macroalgae are commercially and scientifically cultivated (see further the section on Genetic engineering of algae: examples of environmental and industrial applications).

4. Gene Technology

4.1. Polymerase Chain Reaction

The capacity to amplify specific regions of DNA, by using the polymerase chain reaction (PCR), has in many ways revolutionized the molecular biology discipline (see also Physical methods of analysis; Methods in genetic engineering). In PCR reactions specific DNA fragments are synthesized in vitro. The product obtained contains millions of copies of the fragment and can therefore easily be identified and isolated from the rest of the DNA genome. The PCR technology is nowadays used as a routine tool in most molecular studies, including genetic engineering. An important property of the PCR is the capacity to amplify a target sequence from a crude DNA template. This has become very helpful in many applications within the algal field of research. PCR on crude template preparations are very useful in phylogenetic and taxonomic studies on species that can not be obtained in pure culture. The PCR technology has also become irreplaceable within ecological and physiological research.

4.2. Cloning

One of the major problems when applying genetic engineering on new kinds of organisms is the problem to design specific vectors that can both be transformed into the cells, accepted by the cell and expressed in an adequate way. The ability to introduce and achieve desired levels of expression of foreign genes have been made possible by:

1. Technical development for the incorporation of DNA into algal cells. Techniques used in transformation of algal cells include injection of DNA through fine glass needles (microinjection), bombardment of cells with DNA coated gold particles, and virus infection. Other methods used to make the cells prepared for uptake of DNA fragments or plasmids are the use of electrical charge to temporarily open pores in the cell membrane (electroporation) or agitation of algal protoplasts, i.e. algae without cell walls, with glass beads.
2. Development of promoter systems so that the introduced DNA can be expressed by the algal cells in a satisfying way. Homologous promoters are usually

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preferred since heterologous promoters (those from other organisms) sometimes do not drive the expression of the transformed genes in an efficient way.

3. (c) Selection of reporter genes, which identify the cell that has been successfully transformed. In bacteria, genes conferring antibiotic resistance are the most widely used reporter genes. Usually antibiotic resistance genes are not used as reporter genes in algae due to their often- natural resistance to antibiotic compounds. Reporter genes that have been used include the gene that encodes the enzyme arylsulfatase. This enzyme in normally expressed under sulfur starvation and it causes the algal cells to produce an easily detectable coloured substance. Pesticide resistance is popular as selective markers in plant genetic engineering, and can probably also be used as such in similar studies on algae

4.3. Hybridization

Artificial construction of a double-stranded nucleic acid by complementary base paring of two single stranded nucleic acids (RNA or DNA) is called hybridization (see also Genetics and Molecular Biology). This technique has become a powerful tool in genetic research. It also permits the detection of smaller stretches of nucleic acid that are complementary to a known sequence. Such a single-stranded molecule of known sequence is called a probe. A probe labeled with some kind of detection molecule (radioactive, fluorescent or color) can be used to locate a sequence complementary to the probe within a mixture of nucleic acids of unknown composition and origin. Hybridization can be performed both on isolated DNA bound on a matrix support (filter, beads, plastic wells), in solution or directly on preserved whole cells or tissue. Within algal research, whole cell hybridization has been used to distinguish between closely related strains or for the enumeration of a single species within a large assembly of species (i.e. natural water samples).

5. Genetical Identification and Phylogeny

5.1. Origin of Chloroplasts

Photosynthetic eukaryotes were probably generated only once by engulfment of a photosynthetic prokaryote, in spite of the diversity of algae observed today. Prochlorophytes prokaryotes that perform oxygenic photosynthesis using chlorophyll *a* and *b*, have been proposed as the ancestor. The chlorophyll *b* synthesis gene (CAO) has been isolated and sequenced from two prochlorophytes and major groups of chlorophytes. The phylogenetic analyses show that these genes share a common ancestor. This finding suggests that the ancestor of chloroplasts had both phycobilins (the pigments specific for Cyanophyta, Rhodophyta, Glaucophyta and Cryptophyta) and chlorophyll *b* (pigment specific for Euglenophyta, Chlorophyta and all higher plants). This explains the presence of chlorophyll *b* and a type of phycobiline in *Prochlorococcus marinus*, the sole photosynthetic organisms that contains both these pigments. Serial loss of various pigments, and eventually the loss of photosynthesis that led to the conversion of chloroplasts to pure plastids or loss of the entire plastid, further increase the diversity. This scheme explains the close evolutionary relationship between some photosynthetic eukaryotic algae and heterotrophs such as the genera *Dinophysis* and *Phalachroma.*

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5.2. Use of Conserved Genes

Without complete DNA sequence information of every organism, the analysis of small conserved genes has proved to be very helpful in the clarification of the relationships between algae. This has led to a supplementation and in some cases considerable revision of the old determinative classification of algae by a more natural, phylogenetic one. The most common DNA regions analyzed today for phylogenetic purposes are ribosomal RNA genes, mitochondria genes, plastid genes, photosynthetic genes, actin genes, ITS (internal transcribed sequences, e.g. regions between ribosomal and transfer RNA genes) and microsatellite DNA sequences. We will focus on the ribosomal RNA genes since these are most commonly studied.

The major revision of algal classification and phylogeny came with the introduction of techniques for ribosomal RNA (rRNA) sequencing. Ribosomal RNA's are exceptionally useful for the comparative analysis of organisms. They are very slowly altering molecules and major elements in the protein synthetic machinery of all cells. This conserved nature is also a feature on the nucleotide sequence level. Some sequence islands are invariant in all biological kingdoms, which make them ideal as targets for kingdom specific identification probes, or as primer targets in sequencing reactions. Other sequences within the rRNA molecules vary to a greater or less extent. Small rRNA (SSUrRNA) genes are more highly conserved than large rRNA (LSUrRNA) genes, and are therefore more useful for analysis of more distantly related species. Analysis of the LSUrRNA gene has been very useful for sorting out closely related species concepts, like species groups and geographical origin of different clonal isolates (see below, section 5.3).

5.3. Molecular Identification of Algae

A major problem in scientific and monitoring programs is to identify and enumerate the algae of interest. Another crucial problem is that few people are able to name and classify algae. There are several reasons for this. The most significant is that most of these algae are of microscopic size (Figure 5) and in many cases scanning or transmission electron microscopy is necessary to identify algae to species level. Even some macroalgal genera and species are difficult to identify. Many fragile algae do not survive collection and fixation or they shrink, loose pigmentation and flagella so that a proper identification is impossible. Furthermore, some important species constitute just a minor fraction of the total planktonic community, which leads to tedious analysis of discrete samples, a common problem in harmful algae monitoring programs.

An increasing number of researchers are employing different molecular methods to define and enumerate algae. These methods include the development of class, gene or species-specific probes or primers. One example is to use specific rRNA targeted probes that have been labeled with fluorescent molecules and thereafter used in whole cell hybridization experiments. Cells labeled in this way can either be visualized by fluorescence microscopy or sorted and detected by flow cytometry. This technique has been used to distinguish cells of toxin producing diatom *Pseudonitzschia* from similar, but non-toxic species and to identify the nanoflagellates *Chrysochromulina* (Figure 6) and *Prymnesium*.

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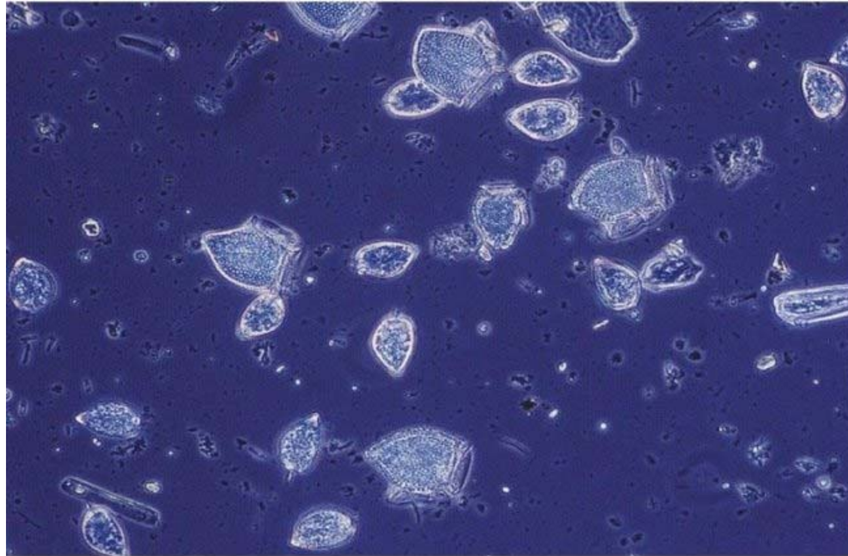

Figure 5. Microscopic view of a natural microalgal community dominated by *Dinophysis spp*., (Dinophyta), Skagerrak, NE Atlantic. (Photo Ann-Sofi Rehnstam-Holm, Clinical Bacteriology, Göteborg University, Sweden).

Other researchers have developed PCR procedures for specific identification of algal species, with the use of species-specific primers, within a background of numerous planktonic species. Using this strategy, *Dinophysis acuminata* could be detected from natural samples by species-specific PCR at concentration as low as 30 cells per liter, and the presence of *Alexandrium tamarense* could be detected in filtered seawater spiked with cultured cells at a DNA template concentration of 2×10^{-13} g μl^{-1}. Genus specific primers together with radioactive labeled probes for *Alexandrium* spp. could detect cultured *A. lusitanicum* at a DNA template concentration of 1.5×10^{-15} g, corresponding to 100 cells and sensitivity analysis of species-specific primers targeted for *A. minutum*, gave a positive PCR signal at a cell concentration of 0.3 cells per litre. These findings suggest that PCR is a specific and very sensitive method for detecting algae in natural water samples. Rapid identification of the fish killing dinoflagellate *Pfiesteria piscicida* in natural samples has recently been possible due to a heteroduplex mobility assay. In this assay, PCR amplified SSUrRNA from a known “driver,” in this case *Gymnodinium sanguineum*, were hybridized to unknown populations of amplified *Pfiesteria* SSUrRNA. When different heteroduplex formations were separated on a denaturing electrophoresis gel, clear clonal patterns were observed and several new environmental isolates of *Pfiesteria*-like sequences were obtained.

Genetic variability within an algal species or species group has become a concern in many studies of taxonomy, population dynamics and biogeography. This is especially true for morphologically very similar species. The most thoroughly studied is the *Alexandrium tamarensis*, *A. catenella* and *A. fundyense* complex. Both the SSUrRNA genes and the highly variable regions in the LSUrRNA genes have been sequenced and phylogenetically analyzed. These analyses revealed several major classes that divided the *Alexandrium* species complex in a pattern that did not correlate to morphospecies label. Furthermore, the different classes could be correlated to regional populations in such a way that species collected from the same geographic region were most similar,

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regardless of morphotype. Geographical isolated strains diverged more significant regardless of high similarity in morphological features.

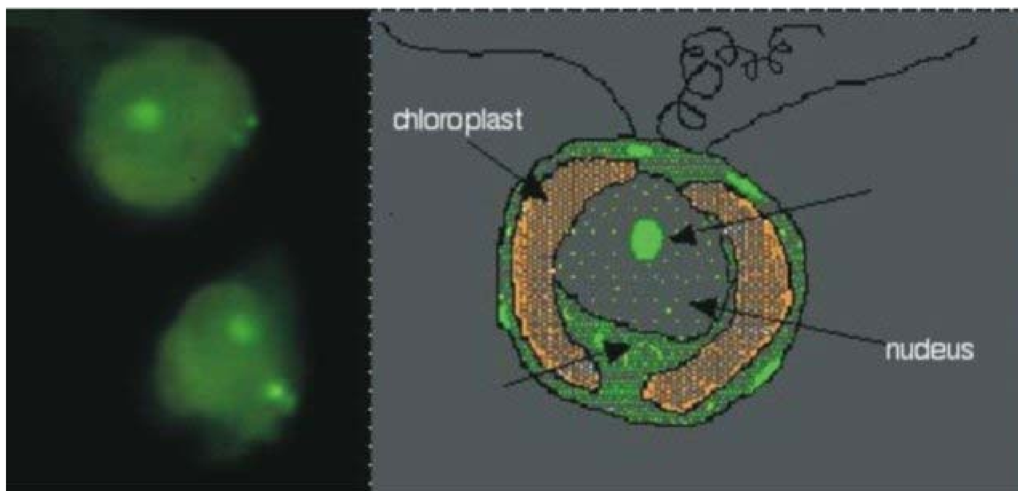

Figure 6. In situ hybridized *Chrysochromulina polylepis*(Haptophyta). Cultured *C. polylepis* was hybridized with a ribosomal RNA targeted fluorescently labelled probe. Notice the brigt light from the cells nucleolus (a region within the nucleus where rRNA is synthesized).[Photo: Ann-Sofi Rehnstam-Holm]

5.4. Molecular Identification of Algal Populations

Ecological studies often depend on accurate assessment of the genetic diversity in a population. DNA or RNA is extracted from environmental samples and various techniques and genetic markers of multi- or singlelocus types are used to analyze the sample diversity. The PCR technique has been used to screen mutations conferring herbicide resistance in natural populations of red algae *Porphyra linearis*. Several samples of *P. linearis* were collected along the mid-Atlantic coast of France and the obtained sequences indicated mutations, but not in known herbicide resistance genes.

A rather new technique that has gained much interest is amplified fragment length polymorphism (AFLP). However, AFLP has found the widest application in analysis of genetic variation below the species level.

Molecular information can also be used to evaluate differential gene expression in natural environments. By analyzing isolated *rbcL* mRNA with probes obtained from a diatom (*Cylindrotheca* sp.) and a cyanobacterium (*Synechococcus* sp.), researchers could show that diatom *rbcL* gene expression appeared to decrease from near shore to off shore and that the cyanobacterial expression did not follow this pattern in samples obtained from Lake Eire. After reverse transcription of *rbcL* mRNA, portions of the various obtained cDNAs were amplified by PCR and sequenced. Distinctly different sequences were obtained from near surface and subsurface samples and this suggested that there was a stratificated situation of active CO_2-fixating organisms in the lake.

Nucleic acid probes can also be used to evaluate changes in carbon fixation rates and how this fixation can vary from one algal species to another. Analyses of two major

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classes of RUBISCO-containing phytoplankton, the cyanobacteria/chlorophytic clade and the chromophytic clade (diatoms, chrysophytes, prymnesiophytes and others) occupied separate niches in time, space and cell size. The majority of the chromophyte *rbcL* mRNA was concentrated at the subsurface level while the cyanobacterial/chlorophytic *rbcL* mRNA was found in the upper water column.

6. Genetic Engineering as a Tool to understand the Physiology, Biochemistry and Molecular Biology of Algae

6.1. Model Organisms

6.1.1. *Chlamydomonas Reinhardtii*—The "Green Yeast"

The green algae *C. reinhardtii* has presented itself as a particular favorable photosynthetic organism for genetic studies. Already in 1918, a paper was published describing the life cycle and Mendelian inheritance based on taxonomic studies for *C. reinhardtii*. Genetic and physiological features of this unicellular alga have provided a useful model for elucidating the function and regulation of nuclear and chloroplast gene expression. Much of the information acquired of the photosynthetic apparatus of plants has been generated through studies of *C. reinhardtii*. This organism is often easier to study than higher plants due to its haploid genome, the short generation time, uncomplicated growing requirements and the fast response to a changing environment. Another feature of high value when studying photosynthetic mechanisms is the ability to grow without light. *C. reinhardtii* can be grown heterotrophically on acetate and it is relatively easy to isolate and maintain photosynthetic mutants that are light sensitive.

Valuable molecular tools have been tailored to be used with *C. reinhardtii*. The most important developments have been the establishment of selectable markers for identification of nuclear and chloroplast transformants and relatively simple procedures to introduce foreign DNA into cells. Moreover, reporter molecules have been developed which allows inserted genes and their products to be localized within the cell. Experiments on *C. reinhardtii* show that transformations of the nuclear genome as well as the chloroplast genome can be accomplished. In the chloroplast genome it is possible to insert DNA at a precise location, whereas in the nuclear genome DNA is integrated randomly, which implies the impossibility to inactivate any precise gene in the nucleus.

Although molecular-genetic studies of cellular processes in photosynthetic eukaryotes using *C. reinhardtii* as a model system has been very fruitful, progress has been hindered by the difficulties to express foreign genes in the nucleus of this organism. This has prevented, for example, the use of heterologous genes to complement mutants or to manipulate key metabolic pathways. Several attempts to find a way to increase the yields of foreign gene expression have been made. During these studies valuable information on the processes of gene expression, transition and translation has been acquired.

In one experiment, a bacterial gene (*ble*) encoding zeomycin resistance was inserted in the *C. reinhardtii* genome. They then followed whether the transgene expression was enhanced when endogenous *Chlamydomonas* introns were fused into the coding region

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of the foreign *ble* gene. The transformation frequency and the level of *ble* expression were stimulated. The improvement was found to be mediated by an enhancer element within the intron sequence.

Another approach to improve the expression of transgenes in *Chlamydomonas* has been achieved by creating a plasmid consisting of a fusion of an endogenous promoter (*HSP70A*) and the foreign gene of interest. The studies have been performed by constructing several different plasmids with different sets of promoters in different frequencies. The RNA yields of the different promoter set-ups were quantified by Northern Blot analyses, and the quantity of translated transgene proteins measured. This showed that the foreign gene expression was enhanced when the gene was fused with an endogenous promoter and which promoter configuration that gave the largest yield of transcript.

In another study, several strains of *C. reinhardtii* were transformed with a fused gene construct, consisting of a bacterial gene conferring spectinomycin resistance, and the regulatory region of an endogenous gene. Although these cells were genetically identical they displayed phenotypic variations. The level of expression of the introduced gene varied from cell to cell and the level of transcription was vegetatively inherited. The nature of repression of this transgene *C. reinhardtii* is believed to be epigenetic, in other words, the inactivation of the gene is not caused by modifications of the inserted DNA sequence but rather on the transcription level.

6.1.2. Cyanobacteria

Several genetic engineering studies dealing with the physiology, biochemistry and molecular biology of algae, have been performed on cyanobacteria. Cyanobacteria is a preferable source material in plant genetic engineering studies due to genetic homology of chloroplasts in eukaryotic plants, prokaryotic genome organization, short generation time, and fast growth.

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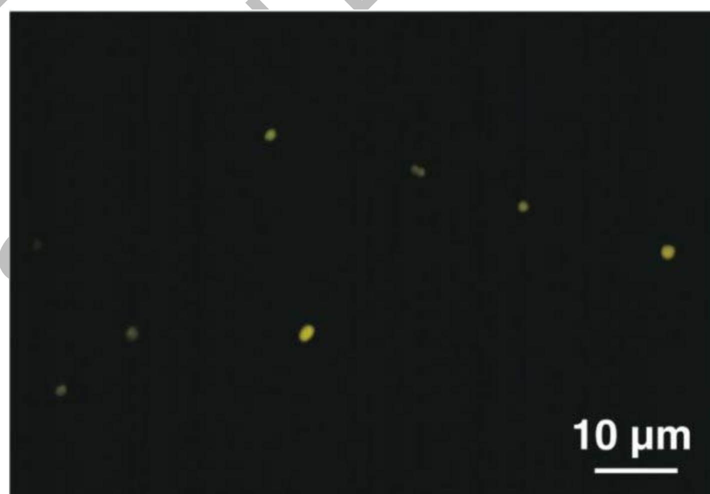

Figure 7. Epiflouroscence micrograph of *Synechococcus sp.*, Cyanophyta, from Skagerrak, NE Atlantic. (Photo: Bengt Karlson, Marine Botany, Gteborg University, Sweden).

Most of the studies of manipulated genes and proteins of the photosynthetic system have, apart from *Chlamydomonas*, been made on cyanobacterial strains of *Synechocystis* sp., *Synechococcus* sp. and *Anabaena variabilis. Synechocystis* sp. and *Synechococcus* sp. (Figure 7) are naturally transformable with exogenous DNA. Furthermore, these two strains can be grown under heterotrophic conditions in a light-stimulated environment, i.e. they must occasionally be exposed to flashes of light. This is a desirable trait for an organism in which mutations in photosynthetic processes are generated. DNA can be introduced into *Anabaena* cells through conjugation transfer and it can grow in darkness in the presence of fructose.Figure 7. Epiflouroscence micrograph of *Synechococcus sp.*, Cyanophyta, from Skagerrak, NE Atlantic. (Photo: Bengt Karlson, Marine Botany, Göteborg University, Sweden).

6.2. Genetic Studies of Photosynthesis

The event of photosynthesis (see also Cell thermodynamics and metabolism) provides most of the available energy to maintain life on earth. Photosystem I and II (PSI and PSII) are two multisubunit pigment protein complexes responsible for this process. PSI and PSII are located in the thylakoid membranes in cyanobacteria and chloroplasts of plants and eukaryotic algae. Both PSI and PSII have specialized chlorophyll molecules in which the absorption of light energy initiates a series of reaction that results in the production of high-energy molecules, such as ATP and NADPH. To understand the processes of photosynthesis, directed mutagenesis in genetically manipulated microorganisms have been used to specifically modify individual polypeptide components of the photosystems in cyanobacteria and the green algae *Chlamydomonas*. Especially PSII has been extensively studied because it harbors binding sites for various commercial herbicides.

The genes and the respective DNA sequences of the PSI and PSII proteins have been unraveled. Directed modifications of the genes or the proteins have revealed the inherent property, function and structure of the proteins and site-specific mutations have been created through generation of interruption or deletion mutants. The effect of the mutation has thereafter been studied on a phenotypic and biochemical manner revealing the consequences of the absence of a certain protein. Even individual residues of the peptides being of extra importance of the function of proteins have been identified. Moreover, it has been found that some proteins of the PSI and PSII are apparently needed for growth and survival although some proteins are still of unknown function since mutants deprived of such proteins continue to grow. Site-specific mutations in genes of this kind have verified or invalidated hypotheses postulated earlier through biochemical studies. By comparing the sequences of the genes encoding different proteins within the PSI and PSII, a theory of the evolution of the complexes has been established.

Mutations leading to inactivation of specific genes in eukaryotes and prokaryotes have revealed similarities as well as dissimilarities in the phenotypic function of presumably homologous proteins. Some functions and structures of PSII have been found to have remarkable similarities to proteins within the reaction centre complexes of anoxygenic purple nonsulfur photosynthetic bacteria. PSI in plants and algae resembles the photosystem of green sulfur bacteria.

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The complete chloroplast genome has been sequenced in some plants and eukaryotic algae. The analyses of the sequences have revealed the presence of a number of unidentified open reading frames (ORF) that may encode proteins functionally important in PSI and PSII. Over several years in the past a systematic investigation consisting of targeted mutations in each and every ORF of *Chlamydomonas* chloroplast genome has been performed. This thorough study will undoubtedly reveal whether the encoded proteins are essential or not for the cell.

Many genes encoding proteins involved in photosynthesis have been isolated through proteins. This means that the amino acid sequence of the protein is determined, and from this amino acid sequence the genetic sequence is obtained. However, this strategy is not appropriate for the isolation of all genes, for example, the genes encoding components for chlorophyll *b* synthesis. The reason for this is that no *in vitro* assay system for chlorophyll *b* formation is available.

When a foreign DNA fragment is inserted in a host genome, it may disrupt a gene. Moreover, the insertion may also be accompanied by a large random deletion of host genomic DNA. This feature has been used in the isolation of genes encoding for chlorophyll *b* synthesis. *Chlamydomonas* cells were transformed with plasmid DNA carrying nitrate reductase as a selective marker (many laboratory "wild type" *Chlamydomonas* strains lack the capacity to use nitrate as the sole nitrogen source due to the lack of nitrate reductase). The resulting strains were found to be chlorophyll *b* mutants created by insertional mutagenesis. The nitrate reductase gene was thereafter used as a marker to obtain the DNA sequences of the genomic regions flanking the inserted nitrate reductase gene, since this was presumed to be parts of the lost chlorophyll *b* gene. This strategy was used in the screening of several mutants and different DNA clones, in respect of the genomic part of the sequence, were obtained. These partially genomic sequences could then be used as new probes and different overlapping sequences were identified from the wild-type *Chlamydomonas* DNA library. By using these methods a region responsible for the loss of the chlorophyll *b* character was defined. The full region was sequenced in wild type *Chlamydomonas* and the amino acid structure of the encoded enzyme was determined. By the definition of the amino acid sequence the enzyme's (chlorophyll *a* oxygenase, CAO) reaction pathway could be established. It was discovered that the CAO product carries out the conversion of chlorophyll a to chlorophyll b alone. All oxygenic photosynthetic organisms that contain chlorophyll *a* can synthesize chlorophyll *b* only by obtaining CAO. It has also been confirmed that the CAO gene is present in only one copy and it is highly conserved.

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Some photosynthetic processes are regulated by an array of different genes. A single mutation may then not be enough to reveal the structure of the processes. Studies of cyanobacteria that possess a CO_2-concentrating mechanism (CCM) that elevates the concentration of CO_2 and thereby enables efficient CO_2 fixation and photosynthesis have indicated that several genes regulate this process. The activation of CCM in cyanobacteria rises when the CO_2-concentration declines. A plasma membrane protein has been demonstrated to increase under CO_2-limited condition. This protein was therefore suggested as responsible for CCM. In a recent study, a *Synechococcus* mutant was obtained that lacked this protein. If the plasma membrane bound protein were the

sole protein needed for CCM, one would expect no CO_2-inducible activity in the mutant. However, even though the mutated strain showed lower inducible CCM activity than wild type *Synechococcus*, it was not totally defected in this mechanism. Hence, the CCM is suggested to be regulated by more than one gene.

6.3. Genetic Studies of Photoprotection

Photosynthesis inevitably generates highly reactive intermediates and by-products that can cause oxidative damage to the photosynthetic apparatus. This photo-oxidative damage is termed photoinhibition, and if not repaired decreases the efficiency and/or maximum rate of photosynthesis. Photosynthetic organisms have evolved multiple photoprotective mechanisms to cope with the damaging effect of light. Adjustment in light-harvesting antenna size and photosynthetic capacity can decrease light absorption and increase light utilization, during acclimatization to excessive light. Alternative electron transport pathways and thermal dissipation can also help to remove excess absorbed light energy from the photosynthetic apparatus. In addition, numerous antioxidant molecules and scavenging enzymes are present to deal with reactive molecules, especially reactive oxygen species.

Genetic and molecular techniques have proved fruitful to dissect specific processes involved in photoprotection. The green algae *Chlamydomonas reinhardtii* and *Scenedesmus obliquus* are common model organisms used to understand the process. Several mutant strains of *C. reinhardtii* affected in the photoprotection in the chloroplasts are available. In these strains different steps are blocked in the carotenoid synthesis, enzymes engaged in the photoprotective processes are over-expressed or the mutation is targeted towards the photosynthetic repair system. Because photoprotective processes comprise several lines of defence against the damaging effects of light, construction of double and sometimes triple mutants may be necessary to obtain clear phenotypes. By constructing mutants lacking one or more steps of different synthesis pathways, the relative importance of the different components, and alternative pathways for photoprotection have been understood. The period needed for acclimatization to a new light environment has also been investigated through phenotypic characters expressed during different culturing conditions. The mutant strains have also revealed that many of the accessory pigments and enzymes involved in the photoprotection have multiple roles. Comparing mutants in specific genes between algae and higher plants have revealed different phenotypic expression although the same gene is mutated.

6.4. Genetic Studies on the Function of Flagellae

The flagellar apparatus and ciliary motion consist of an array of complex proteins. Attempts to identify and characterize the flagellar apparatus have earlier been done by ultrastructural and biochemical techniques on *Chlamydomonas reinhardtii* mutants. With the tool of genetic engineering, studies of flagellar functions have progressed through insertional mutagenesis. Wild type strains have been transformed with plasmids encoding a gene used as a selective marker. The genomic sequences flanking the integrated plasmid of the mutant has then been compared to wild type sequences, and the genes lost by the insertion have been characterized. The phenotypes of the mutants have been studied and by rescuing the mutants (with a new transformation with a

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plasmid containing the excised gene) the relation of genes, encoded proteins and functional and structural properties of flagella have been established.

In immobile mutants of *C. reinhardtii*, a gene encoding a crucial dynein protein for flagellar movements has been isolated and identified. Analogous genes (and their proteins) have been identified in sea urchins and the green alga *Spermatozopsis similis*. These findings have shown that the genes coding for the crucial flagella and cilia proteins, are evolutionary very conserved.

Primary Ciliary Dyskinesia (PCD) is a human syndrome characterized by severe respiratory disease associated with male infertility due to lack of flagellar motion of sperms. The main ciliary defect in PCD is the lack of dynein protein arms. Could similar mechanisms be responsible for immobility in *C. reinhardtii* and the human syndrome PCD?

To show if this was the case the most conserved regions of the genes encoding the dynein protein in *C. reinhardtii* and sea urchin were used to construct primers. By using these primers the human gene (*DNAI1*), encoding a homologous dynein protein responsible for ciliar movements in humans, could be isolated and characterized. Mutation in *DNAI1* verified the connection between the gene and the human PCD syndrome.

6.5. Genetic Studies on Transport of Proteins into Plastids

Genetic engineering has proved to be a successful tool in the studies of protein transport within the cell. This subject is very well studied on higher plants, whereas less has been done to understand the protein transport mechanisms within the cell in algae.

A gene from the cryptophyte *Guillardia theta* was sequenced and characterized as coding for a specific protein. Thereafter DNA was transcribed and RNA translated into protein *in vitro*. The biochemical structure of the protein and the precursors could thereafter be studied and the protein was labeled and transported into isolated plastids of the same species and higher plants. Similar studies have also been conducted on protein transport in the diatom *Odontella sinensis*.

6.6. Markers used for Growth Studies

The obligatory photoautotrophic green algae *Volvox* sp. is one of the simplest multicellular organisms known, consisting only of two types of cells, somatic and reproductive. Hence, *Volvox* is a valuable model when studying early mechanisms of cell differentiation. Biochemical studies of development processes in an organism require an efficient procedure for metabolic labeling of relevant molecules. The incorporation of radioactive ^{14}C, in sugars, for example, is crucial to identify and study the developmentally controlled synthesis of unknown proteins, glycoproteins, lipids and carbohydrates. *Volvox*, being a photoautotroph does not incorporate any organic carbon from the environment, and hence lacks import systems for sugars and amino acids. The hexose/H^+ symporter gene coding for glucose transporting proteins from the conditionally heterotrophic unicellular alga *Chlorella* has been cloned into and

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expressed in *Volvox*. In transformed *Volvox* the incorporated ^{14}C macromolecules could be studied and the transgene *Volvox* exhibited prolonged survival in the dark, and in the presence of glucose. This system might very well be used as a selectable marker in other photoautotrophic organisms.

6.7. Processes Regulated by the Circadian Clock

Many metabolic and behavioural mechanisms follow a circadian rhythm, i.e. a cycle of approximately 24 hours. A circadian clock at the translational level regulates the luciferin-binding protein (LBP), which is causing bioluminescence in, for example, the dinoflagellate *Lingulodinium polyedra*. Bioluminescence is reaching its maximum at the middle of the night phase and an RNA-binding, circadian controlled translational regulator (CCTR) protein controls this. CCTR decreases its binding activity at the beginning of the night phase when the synthesis of LBP starts, and increases again at the end of the night. This suggests that CCRT function as a clock-controlled repressor.

The site of the mRNA where this circadian clock-controlled protein binds has been identified to be located in a flanking repetitive region. By designing a probe for this region, a CCTR analogue was identified for the distantly related *Chlamydomonas reinhardtii*. The RNA-binding protein identified from *C. reinhardtii* has been denoted as Clamy 1. It has also been demonstrated how many of the units that are required for the protein to bind by systematic elimination of repetitive units using site directed *in vitro* mutagenesis. Both the clock-controlled proteins CCTR and Clamy 1, required the same number of repetitive units and the loss of binding capacity of the proteins were of the same range in the two species.

7. Genetic Engineering of Algae: Examples of Environmental and Industrial Applications

7.1. Cyanophyceae as N-fertilizers and Bioremediators

Cyanobacteria possess the unique ability among photosynthesizing organisms to fix nitrogen directly from the atmosphere and to convert N_2 into nitrogen compounds that can be biologically incorporated. The cyanobacteria form a prominent component of the microbial populations of wetland soils, especially in rice paddy fields, substantially contributing to fertility as a natural biofertilizer. By using $^{15}N/^{14}N$ tracer techniques it has been shown that nitrogen from nitrogen fixating cyanobacteria is directly transferred to rice plants, and around 40 percent of the cyanobacterial nitrogen has been found to be recovered in rice plants. Modern agricultural fields that are generally treated with high doses of synthetic nitrogenous fertilizers and pesticides, have adverse effect on the naturally occurring N_2-fixing cyanobacteria. The ability to fix nitrogen is reduced due to lowered nitrogenase activity (the group of enzymes responsible for nitrogen fixation) in the presence of external nitrogen. It would therefore be of great advantage to develop pesticide resistant cyanobacteria that also can fix atmospheric N_2 in the presence of external nitrogenous fertilizers. Genetically manipulated cyanobacterial species may act as the self-renewable constitutive N-supply, producing ammonia at the sole expense of CO_2, N_2, solar radiation and water, irrespective of the pre- or post treatment of the fields with pesticides and/or fertilizers. These genetically engineered species may not

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completely replace the use of fertilizers but could contribute in considerably reducing the use of synthetic N-fertilizers.

Mutations in the genes coding for enzymes involved in the pathway of ammonium assimilation, have been shown to cause high levels of nitrogenase synthesis even in the presence of ammonium. Paddy cultures with these genetically improved cyanobacterial mutants are 40 to 45 percent more efficient, as compared to results obtained with the wild type cyanobacteria.

Herbicide-resistance in various strains of cyanobacteria has been achieved through either mutation or genetic engineering. Genetically engineered strains consist of originally sensitive cyanobacterial strains that have been made resistant through the transfer of resistance genes from other cyanobacteria. A few strains of cyanobacteria are displaying high stable resistance to a specific herbicide along with the acquisition of the capacity to continue performing the function of constitutive nitrogen fixation with no repression from the combined N source present in the exterior environment.

Pesticide resistant cyanobacteria are not only important for thriving in chemicalized agricultural fields and performing constitutive nitrogen fixation, but could also be used as markers in scientific genetic engineering studies, since herbicide resistance is popular as selective markers in plant genetic engineering. A future prospect is that herbicide resistance genes may be transferred from cyanobacteria to the chloroplasts of crop plants.

Bioremediation of contaminated soils are at present a widely used process where heterotrophic bacteria are used to degrade a range of pollutants. However, surface water contaminated with synthetic chemicals remains largely untreated by remediation programs. The advantages to use cyanobacteria for bioremediation in surface water are numerous. Bioremediation by cyanobacteria means lower costs compared to heterotrophic bacteria, since cyanobacteria are independent of an external organic carbon source. Cyanobacteria are also able to degrade target pollutants to a very low level and in addition, the possibility of combining aerobic and anaerobic degradation within one organism exists, since filamentous nitrogen fixating cyanobacteria maintains aerobic metabolism in their vegetative cells and anaerobic conditions in their heterocysts.

Lindane is a commercial insecticide that has been used world-wide since 1940, and is still used extensively in developing countries, especially in rice paddies. The pesticide persists in the environment and can be detected in the air, rain and surface water long after its application. Long persistence of lindane in surface water leads to piscicidal effects or accumulation in fish and is threatening to human health.

Several strains of cyanobacteria are able to degrade lindane (see also Biodegradation of Xenobiotics; and Microorganisms as Catalysts for the Decontamination of Ecosystems and Detoxificatoin of Chemicals). By mutating the nir operon, a DNA region encoding enzymes for nitrate utilization, in wild type cultures of the cyanobacteria Anabaena sp., it has been shown that nitrate is essential for lindane dechlorination. However, in two species of cyanobacteria, Anabaena sp. and Nostoc ellipsosorum, the degradation of

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lindane was enhanced after the incorporation of a lindane dechlorination operon from the bacterium *Pseudomonas paucimobilis*. Dechlorination of the genetic engineered strains became uncoupled from nitrate requirement.

Halobenzonates, especially chlorobenzonates, are common in paper-mill runoffs and are released into the environment by paper and pulp processing industries. These industries sometimes collect the waste streams in sewage ponds, from which the water is recycled. Wild type cultures of cyanobacteria are not able to degrade halobenzonates, but this ability can be introduced after genetic engineering. Catabolic genes responsible for the degradation of halobenzonates have been identified and isolated from the bacteria *Arthrobacter globiformis*. These genes have been cloned into two species of cyanobacteria, *Anabaena* sp. and *Nostoc ellipsosorum*. The cyanobacteria was supplied with an operon from *Arthrobacter globiformis* and could thereafter dechlorinate 4-CB (4-chlorobenzonate) and 4-IB (4-iodobenzonate).

Further studies of genetic engineered algae and bioremediation have been performed on the green algae *Chlamydomonas reinhardtii*. Algae in heavy metal contaminated environments evolve different biochemical strategies to reduce the toxicity. *Chlamydomonas* sp. synthesises heavy metal binding phytochelatins. To further increase the heavy metal binding capacity of the algae a foreign metallothionein (MT-II) gene was expressed in *C. reinhardtii*. Cultures that expressed the MT-II gene absorbed twofold more Cd than wild type cultures.

7.2. Commercially Attractive Compounds from Algae

Astaxanthin is an accessory pigment produced by algae. Only a few animals can synthesize astaxanthin *de novo* from other carotenoids and most of them acquire it by their food. Astaxanthin occurs naturally in some species of cyanobacteria, lichens and algae. Astaxanthin is commercially used as food supplement in aquaculture of fish and other marine animals. It is also a desirable and effective non-toxic coloring for the food industry and is valuable in cosmetics. Astaxanthin has been shown to be an extremely efficient antioxidant that provides protection against oxygen free radicals, it act as an anti-cancer agent and stimulates the immune system. It would therefore be of great economical value if the genes responsible for astaxanthin synthesis could be transferred and expressed in an organism that are easy to grow on a commercial scale (see also Pharmaceuticals from Algae).

The green alga *Hematococcus pluvialis* naturally accumulates large amounts of astaxanthin when exposed to unfavorable growth conditions. The gene coding for the enzyme that converts ß-carotene into astaxanthin has been identified from *H. pluvialis* and cloned into *Synechococcus.* This cyanobacteria does normally convert beta-carotene into zeaxanthin. After the transformation both zeaxanthin and the attractive compound astaxanthin was produced.

Isoprenoids are a large and varied group of organic compounds built up of 5-carbon isoprene units. The fundamental biological functions performed by isoprenoids ensure that they are essential for the normal growth and development processes in all living organisms. These include the function as eukaryotic membrane stabilizers (sterols),

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plant hormones (gibberellins and abscisic acid), pigment for photosynthesis (carotenoids and phytol side chains of chlorophyll) and carriers for electron transport (menaquinone, plastoquinone and ubiquinone). Many of the isoprenoids are of high economic value. An increased isoprenoid synthesis in *Escherichia coli* could be utilized in the industrial production of isoprenoids from bacteria.

Isopentenyl diphosphate (IPP) acts as the common five-carbon building block in the biosynthesis of all isoprenoids. The first reaction of IPP biosynthesis in *E. coli* is the formation of 1-deoxy-D-xylulose-5-phosphate, which is catalyzed by an enzyme called DXPS coded by a *dxps* gene. The *dxps* gene from the cyanobacteria *Synechocystis* sp. has been sequenced and cloned into a plasmid. Cells of *E. coli* were transformed with the plasmid and the *dpxs* gene was expressed. The enzyme activity of DXPS was enhanced twofold compared to untransformed strains of *E. coli*. The production of two end products of the isoprenoid pathway, lycopene (a carotenoid pigment) and ubiquinone (a major component of the aerobic respiratory chain) was increased in the transgene strain of *E. coli*.

The enzyme glucose oxidase catalyzes oxidation of D-glucose and is widely used in analytical biochemistry and food industry. The applications include: conversion of glucose to glucono-delta-lactone (an acidifying agent in cheese production); removal of glucose from food; production of hydrogen peroxide; removal of molecular oxygen from foods and pharmaceuticals.

A functionally related enzyme, hexose oxidase, which has wider substrate specificity, has been isolated and purified from the red alga *Chondrus crispus* (Figure 8).

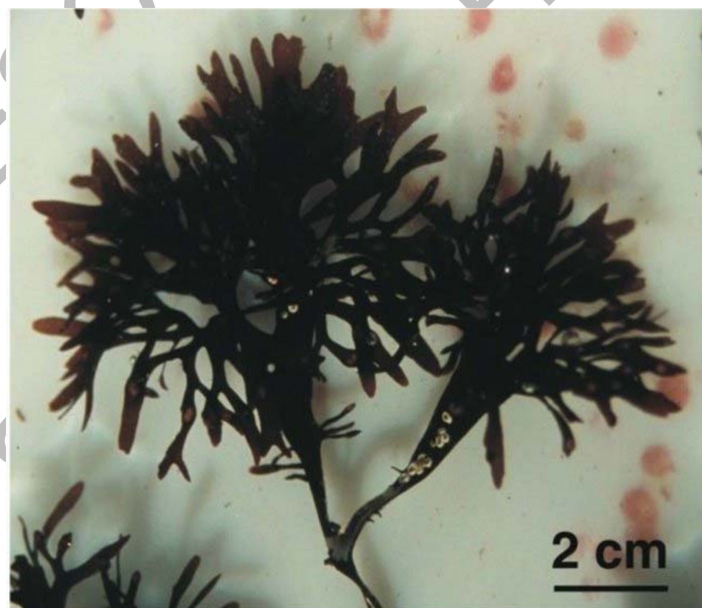

Figure 8. *Chondrus crispus* (Rhodophyta), from Tjärnö, west coast of Sweden. (Photo: Annelie Lindgren, Marine Botany, Göteborg University, Sweden).

The wider substrate specificity of hexose oxidase might provide a greater applicability. This enzyme catalyzes the oxidation of a variety of mono- and disaccharides including

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D-glucose, D-galactose, maltose and lactose, with concomitant reduction of molecular oxygen to hydrogen peroxide. The purified enzyme from *C. crispus* has been cleaved and the peptide fragments have been subjected to amino acid sequence analysis. DNA oligonucleotides have been designed on the basis of amino acid sequences and cDNA encoding *C. crispus* hexose oxidase obtained. The amount of the enzyme is very low in red algae and therefore the gene encoding hexose oxidase has been cloned and expressed in the yeast, *Pichia pastoris,* which can be grown in an industrial scale.

7.3. Cultivation of Marine Macro Algae

The brown alga *Laminaria japonica* is originally introduced to China from Japan and is there cultured extensively. In China, there is no natural population of this kelp due to high summer temperatures. Genetic breeding of *L. japonica* has been conducted since the 1950's and has created a few highly productive strains. This has increased the annual production from 10 tonnes dry weight in 1952 to current 350 000 tones. *L. japonica* is consumed as a subsidiary food and used in the production of iodine mannitol and alginate (see also Marine biotechnology).

Genetic engineering is expected to be an effective mean to develop kelp as a marine bioreactor to produce oral drugs such as vaccines. Yet, no successful transformation in kelp has been reported but models for the insertion of foreign genes have been set up. The model includes introduction of foreign DNA by biolistic bombardment, in other words bombarding cells with microprojectiles coated with DNA and the use of the SV40 promoter as a transcription initiator to drive the gene expression. Moreover, it has been demonstrated that successful transformations are most likely when female gametophytes act as gene hosts, parthenogenesis is the regeneration route and chloramphenicol is used as a selectable reagent.

Extra precautions are needed to prevent transgenic plants from escaping when genetic modified organisms are grown in environmentally open enclosures. It is suggested to use containers with permeable membranes to get a proper water flow. To avoid the release of spores through the membranes of the containers it is necessary to harvest the kelp before the formation of sporangia and to collect the gametophytes indoors.

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Glossary

Bioluminescence: The production of light by living organisms.

Bioremediation: Removing pollution from environment by means of biological organisms.

Conjugation: In unicellular organisms, the joining together and exchange, or one-way transfer, of genetic material.

Cytometry: Counting cells, flow cytometry- a machine counting cells in a

fluid.

DNA: Deoxyribonuclic acid. The hereditary material of all cells and some viruses.

Endosymbiosis: An organism living inside the cells of another in a symbiotic relationship.

Eukaryote: A cell possessing a membrane-enclosed nucleus and usually other organelles.

Heteroduplex DNA: DNA duplex comprising two strands of different origin.

Intron: A non-coding nucleotide sequence, which interrupts the coding sequence in eukaryotic genes and is transcribed, but removed by RNA splicing to leave a functional mRNA.

In vitro: Outside the cell.

Operon: A genetic unit in bacteria, in which several genes coding for the enzymes of a metabolic pathway are clustered and transcribed together.

PCR: Polymerase Chain Reaction. A method used to amplify a specific DNA sequence *in vitro* by repeted cycles of synthesis using specific primers and thermostable DNA polymerase.

Phylogeny: The evolutionary history and line of descent of a species or a higher taxonomic group.

Piscicide: Chemical compound toxic to fish.

Plasmid: Small self-replicating circular DNA independent of the chromosome.

Plastid: A cellular organelle containing pigment, e.g. chloroplasts in plants.

Primer: Short synthetic nucleotide sequences used in PCR reactions.

Prokaryote: Bacteria (Eubacteria and Archaebacteria). Cells lacking nucleus and other organelles.

Promoter: DNA region involved in, and necessary for initiation of transcription.

Rubisco: An enzyme responsible for CO_2 fixation in the process of photosynthesis.

Transformant: A cell that has undergone artificial insertion of genetic sequences.

Transgene: Any gene introduced into an animal or plant artificially by the technique of genetic engineering.

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to off shore and that the cyanobacterial expression did not follow this pattern in samples obtained from Lake Eire.]

Biographical Sketches

Ann-Sofi Rehnstam-Holm: Associate Professor, PhD Umeå University 1995, Microbiology.
Research interests: Dinoflagellate molecular phylogeny, toxicity and ecology; molecular studies on a dinoflagellate parasite (*Parvilucifera infectans*); fate of microbes in mussels.

Anna Godhe: M. Sc. Marine Biology. Ph.D. student Marine Botany, Göteborg University. Research interest: Ecology, taxonomy and life-cycle studies of dinoflagellates; general phytoplankton ecology.

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