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1 Introduction

This deliverable is combining the work done in two tasks of the Aquafuels project: a technological assessment of the major bottlenecks in cultivation, harvesting and extraction of biofuels and an overview and description of the possible valuable byproducts that increase the overall value of algal biomass.

The technological assessment gives a brief overview of the current state of the art and indicates where the main technological bottlenecks are that remain to be overcome in order to make large scale utilization of algal biomass for biofuels economically feasible. The main process steps cultivation, harvesting and extraction are discussed separately for microalgae and macroalgae since there are intrinsic differences between them on all aspects of the process. Finally the technological aspect of the refinery into high quality fuels is discussed.

In the second part an overview of byproducts from both micro- and macro algae is given and their applications are discussed. High-value byproducts increase the overall value of algal biomass and increase the economical potential of algae as a multipotent resource for a biobased economy. Much like crude oil is refined in both fuel and fine chemicals, the value of algae is greatly increased if the parts of the biomass that cannot be converted into fuels are utilized for food, feed, chemicals, cosmetics, biomaterials or even pharmaceutical applications.

2 Technological assessment - Major bottlenecks

Similar to deliverable 3.1 the major limitations to future development of algae technology differs quite a lot between the different sources of aquatic biomass. Therefore in the first two chapters the aspects, cultivation, harvesting and extraction are described per biomass source. The last aspect the quality of biofuel product will be discussed separately.

2.1 Microalgae

Large scale cultivation

Commercial biodiesel production from microalgae will only be feasible in large scale production facilities based on highest possible productivity with minimal capital costs. Most experience with production of algae at larger scale under outdoor conditions for longer periods of time is based on cultivation in raceway ponds or in natural lagoons. It is generally accepted that algal biomass production at today's productivities can only be achieved at the low prices required using such raceway ponds, although technological advancements in the young field of photobioreactor research make the gap between open ponds and bioreactors increasingly smaller, as recently illustrated by Norsker et al. (2010). A recent analysis demonstrated that ponds based on simple unlined clay ponds can produce competitive energy (biogas or biofuel) if at the same time waste water is being treated (Lundquist et al. 2010). Since such cultivation is limited to a few locations worldwide, lined ponds will be necessary which will at least double the investment costs with 1 m² of suitable pvc liner costing \$ 15.- (Ben Amotz). This cost alone may make investment costs prohibitively high for profitable biofuels production. Thus, improved as well as cheaper and more durable materials may need to be found to reduce capital costs, while at the same time productivity will have to be increased to the maximum possible. Improvement of their design (mass transfer and light availability) and operation (control and management) can help also to the enhancement of the productivity in these systems. The same is true to a much greater extent for cultivation in so called photobioreactors. Ideal photobioreactors require lowering investment and operation costs and energy use while improving productivity and scalability. More factors however, might influence the microalgae culture productivity, such as: choosing the best location for algae cultivation concerning its environmental conditions (it is indispensable to have the most hours of sun as possible and mild temperatures,), the proximity to water and a carbon source, in order to reduce the transport costs.

Design of photobioreactors is not easy as a large surface to volume ratio is required for efficient supply of solar light. At the same time the high surface to volume makes scale up difficult in respect to mass and heat transfer. On top of that the solar conditions change continuously. Many types of photobioreactors have been developed and probably many more will still be developed. For evaluation of designs it would be important to compare performance of the different systems under the same operational conditions for longer periods of time. In the feasibility analysis we showed that photobioreactors could become

competitive if investment costs are reduced significantly. Investment costs should be less than 15 €/m² (Schenk *et al.* 2008). Similarly investment costs of tubular photobioreactors would have to be reduced to a similar range (Acien, roundtable). Their optimization can be reached through the optimization of the plastic materials used in the photosynthetic area; allowing a good light efficiency, reducing the culture flow friction, lower water use, no direct evaporation losses (CO₂ cannot escape to the atmosphere) with low costs. Although the technological investment costs are relevant for a sustainable production, it is also important to reduce the operation costs, and others costs, namely:

- The selection of the land resources (land costs, harbor proximity)
- The correct adjustment between nutrient supply/availability in the culture and the microalgae nutrient requirements
- Feeding the algae with CO₂ produced by a GHG emitter, contributing to the environment (although marketing the byproducts from industrial CO₂ grown microalgae maybe be a bottleneck)
- Mixotrophic culture, assuming that this process has a positive economical balance
- Optimizing the light distribution in the culture (light supply efficiency), specially in vertical scale-up
- To determine the average temperatures in which algae can growth (optimal temperature represents higher biomass production)
- Recirculation of the culture medium in order to reduce the water and nutrients consumption

Thin plastic film bioreactors are presently developed for this reason. Similarly, disposable flat panel reactors developed by UNIFI and BGU may represent significant investment cost reduction potential, though running costs (pumping, aeration, cooling or heating) may remain significant. Norsker *et al.*, (2010) performed an economical analysis of the different technologies concluding that reduction of investment cost by using open raceways do not compensate the lower productivity achieved in these reactors with respect to closed photobioreactors, thus the use of these last being the best option to produce microalgae biomass. However, it is generally agreed that today's photobioreactor technologies require more energy for construction and operation than they can return in the form of growing biomass (Stephenson *et al.* 2010) (Jorquera *et al.* 2010) (see D1.4; task sustainability), so that dramatic reductions in energy consumption and built in energy will need to be achieved for Photobioreactors to become an option for microalgal biofuels production.

Another important aspect to take into account in the scale-up of microalgae production processes is the control and automatization of the facility. Currently the microalgal biomass market produces about 5 kt of dry matter/year (Pulz and Gross, 2004) whereas to replace 5% of US demand of fuel for transport it is necessary to produce about 65000 kt of oil rich biomass (Chisti, 2007). To perform this scale-up it is necessary to implement automatization and control systems that allows the stable operation of the processes and their optimization. With this not only the manpower required for the operation of the plant will be reduced but also the uptake of nutrients and energy (García-Sánchez *et al.*, 2003). The utilization of advanced control procedures, similar than used in other industries will be required.

Regarding the last issue, Lundsquist *et al.* (2010) referred that in order to produce algae biofuels in a sustainable way production may not waste nutrients in the process. They must be recycled to produce more algae, and the author also refers that efficiencies of more than 90% can be assumed. Finally, the author states that the recirculation of nutrients allows the use of agricultural fertilizers in microalgae production, maintaining the financial viability of the process.

Other technological aspects might be considered such as:

- the utilization of genetic approaches to increase algae solar conversion efficiency (algae strains with low antenna pigment content); (on the downside: byproducts from GMO may be hard to be marketed.)
- methods that might enhance/promote the algae biomass and the subsequent oil production (i.e. nutrient limitation)
- genetic modified algae (GMOs)
- the control of culture biology during biomass algae production (reducing the contamination flora i.e. bacteria, fungi and viruses by cultivating at condition that are unfavorable for these organisms)

At the moment there is no or hardly any experience with production of algae at larger scale under outdoor conditions for longer periods of time, the most prominent examples are outdoor production of *Chlorella* in Asia or *Spirulina* in USA for high value products, but the scale used here is not sufficient to produce significant amounts of biofuels. For this reason it is very important not only to do research at laboratory scale but also develop pilots evaluate and compare their performance as a basis for design of demonstration scale facilities. An example of this is the AlgaeParc project that was recently launched in Wageningen, The Netherlands (www.algaeparc.com). In addition to this, developments in the microalgae field are mainly driven by end users. For the end users there is presently a limit in supply of biomass to develop further processes. Only for this reason some production capacity needs to be realized. With that products like biodiesel from algae can be developed, tested and additional to that a biorefinery program can be developed to obtain more products at once.

In order to facilitate quick development of cultivation technology research at laboratory scale, pilot scale and demonstration scale should run parallel. From importance is also a good exchange of information such that technology developed in the laboratory can be tested under realistic conditions and research at laboratory scale can be done to encounter the problems at large scale. Recently, Lundsquist et. al. (2010) published a financial and economical analysis of microalgae cultivation for biofuels, the authors concluded that based on their technological assumptions, the process has inherent high costs if it is design primarily for biofuels production. Extensive R&D to promote technological advances is still necessary in order to turn biofuels from microalgae a cost competitive product. One mid-term realistic option is the culture of microalgae to produce proteins, vitamins, pigments, etc, as primary product, and biofuels as secondary products or to look into cheap technologies for heterotrophic growth of algae on (waste) feedstocks with an appropriate carbohydrate content.

- Harvesting

After production, the biomass needs to be harvested, the lipids extracted and the remaining cell components need to be recovered. Large microalgal species such as *Scenedesmus* or *Haematococcus* may be harvested by sedimentation alone, a technology that was used successfully at Algatech Qetura until recently for harvesting of *Haematococcus* biomass. Harvesting of small microalgae can be expensive due to high energy requirements and capital costs involved. Because most microalgae are small individual cells (3-30 μm diameter), centrifugation is often used as a preferred harvesting method. However, as the biomass concentration is generally low (< 3 g/L), centrifugation of diluted streams requires a large capacity of the centrifuge, which makes the process energy-demanding and expensive. The same happens with filtration, which has high energy demand, high investment cost and a great operation cost associated with the

consumables required. No single harvest method may be suited to every case. Recovery of the biomass from the broth has been claimed to contribute 20–30% to the total cost of producing the biomass. Actually, any process to harvest microalgae cultures will have high energetic demands due to the great water content in the culture. Thereby, it becomes advantageous to use a step of pre-concentration of biomass, which will relieve the burden of the next operation.

Flocculation, followed by sedimentation and flotation, prior to centrifugation or filtration has been successfully demonstrated (Boussiba et al 1987, 1988) and can significantly reduce harvesting costs and energy requirements, although costs for chemicals and environmental problems with disposal of the effluent may arise. Ideally, algae would flocculate spontaneously at a certain stage of the process or flocculate in the presence of small concentrations of other algae or bacterial species (bioflocculation).

A further consideration in selecting a suitable harvest method is the acceptable level of moisture in the product. Too much moisture in the harvested biomass can substantially influence the economics of product recovery further downstream, if dehydration of the biomass is required after harvest. Because thermal drying is more expensive than mechanical dewatering, thermal drying should be preceded by a mechanical dewatering step such as filtration or centrifugation.

Extraction

After harvesting the needs to be extracted from the cells. First the cells are disrupted and then the oil can be extracted with organic solvents or with green, but more expensive solvents (e.g. supercritical CO₂). The cheapest possible extraction method would be separation of oil based on centrifugation after cell lysis, a process discussed but not yet adequately defined and definitely an area for future research (Lundquist et al. 2010). Most microalgae strains are in general relatively small and have a thick cell wall. For this reason very harsh conditions need to be used (e.g. mechanical, chemical, physical stress, enzymes) in order to break the cells for extraction of the products. This may affect the functionality of cell compounds like proteins which could make continuous processes and biorefinery a challenge. Excretion of the oil by the algal cells, in similarity to what naturally occurs in the microalgae *Botryococcus braunii* will lead to a simplified biorefinery, and improve the downstream economics. However, it will not provide a complete solution since the remaining cell components still need to be recovered from the cells. Thin cell membranes, strong enough to prevent shear damage during production, would facilitate cell disruption. Small spherical cells with a thick cell wall, like *Nannochloropsis*, are clearly not the ideal algae for this reason.

2.2 Macroalgae

Large scale cultivation, bottlenecks and known unknown.

It is safe to say that with at least 60,000 Ha of sea surface cultivated and harvested in China and Japan, large-scale cultivation has proven to be feasible (Bird, 1987). The FAO reports a total of 15,781,159 t of macroalgae cultivated worldwide, with large-scale cultivation of macroalgae currently in place in various Asian countries. The main producers being China (9,933,785 t), Indonesia (2,145,061 t), The Philippines (1,666,556 t), South Korea (921,024 t), Japan (455,400 t), North Korea (444,300 t) and Malaysia (111,298 t). The main market for the algae produced being food and food texturant.

However according to the same report, Europe cultivates only 47 t of macroalgae (between France and Spain). It is still an early stage for cultivation in Europe, and unless demand increases, whether related to food or energy. There is an increasing demand for renewable energy set by the European commission for What are the factors hindering the development of seaweed cultivation in Europe?

-The current multifactorial low demand for indigenous seaweeds is the main factor hindering the development of seaweed aquaculture in Europe. When demand increases, as the main harvesting beds are already being harvested, the standing stock no longer remains a sustainable options and the production should shift toward cultivation on artificial substrate.

-The somehow erroneous perception of Algae as a form nuisance (probably related to blooming events), however latest development and news (whether based on scientific evidences or not) have lead to consumer awareness of the benefits of seaweeds as food, fertilisers, and source of bio-actives.

-The delocalisation of the phycocolloids industry (Agar, Carrageenan, Alginate) to South East Asia and South-America (Bixler & Porse, 2010) have reduced the need for primary production in Europe. The majority of the seaweed aquaculture in Europe is focused on low-volume/high-value species for food, cosmetic or pharmaceutical market or linked to environmental preoccupations (e.g. cultivation of *Asparagopsis armata* for antimicrobial and antibacterial uses in cosmetic, Mata *et al.*, 2010). Both South East Asia and South America are offering competitive prices, low labour costs and simple and intelligent maricultural techniques that have proved to be very successful. The labour intensiveness of seaweed mariculture and the absence of a ready market have been the main reasons why seaweed aquaculture has not developed to any great extent in the west. If seaweed-based cultivation is to develop in Europe and north America, we must look at the market potential of seaweeds and the various ways in which seaweed mariculture can be improved so as to reduce the labour content (Guiry, 2011).

-The availability for suitable site for seaweed aquaculture. A variety of biotic and abiotic factors are required in order to set up a seaweed farm. The choosen site must fulfill the requirement of the target seaweed species as well as the availability of space and competition with other groups and coastal resource users (e.g. shellfish and finfish farmers, fishermen, shipping, yachting, tourism, protected areas) (Werner *et al.*, 2004).

-Nutrient supply. The scale of the cultivation site is such that nutrient is an issue. Upwelling systems have been investigated in California and Japan, pumping nutrient-rich waters from the deep ocean to the surface where cultivation takes place. In other countries, the practice of spraying nitrogen solution has proven to be both expensive and unsustainable.

-Research gaps for large-scale cultivation and its environmental impact. Despite a general consensus supporting an environmental benefit of macroalgal biofuel, there is a paucity of data regarding the environmental impact of large-scale aquaculture and research programmes need to be implemented (refer to deliverable 3.1 research needs). Very little is known about algal pathogenes (Gachon, 2008) and the impact of pest and diseases must be examined as it is a serious matter to large-scale cultivation.

In a chapter dedicated to kelp cultivation, Tseng (1987) described some of the problems occurring in China. The achievement of high productivity (60 T DW/Ha or 500 T WW/Ha) relevant to energy production was the result of large amount of sporelings, labor and material, hence a problematic high productivity cost. At

the time, he also expressed his concerns about the future higher cost of labor and the need for a transition to mechanization. He mentions the diseases occurring with the large-scale cultivation, the need for high productivity strains and the destructive effects of climatic conditions on cultivations. His conclusions are worthwhile quoting “ the human race must make use of the vast ocean sea to yield more products for its benefit and a new era for the mariculture of seaweed for biomass will definitely come sooner or later”. Since then, China has surely demonstrated its leading role in the large scale cultivation of macroalgae, hopefully European aquaculturists and decision makers will use the Chinese experience as a base to develop large scale cultivation. However, Scientists and Industry protagonists will have to demonstrate and achieve low environmental impact in order to become a successful story.

Harvesting

Cultivation In Europe, the lack of large-scale seaweed aquaculture means that there is almost total inexperience with regard to harvesting practice, and therefore specific bottlenecks are largely unknown at present. Macroalgae cultivation must be scaled-up to a sufficient size that technological innovations can be tested at a realistic scale. In itself, this is perhaps the largest bottleneck at present. There are good options for technology transfer from other aquaculture sectors, particularly those that harvest from longlines (e.g. rope mussel cultivation), and that are likely to be readily available adaptable to seaweed cultivation (Dave Millard, BIM; personal communication). In comparison to SE Asia where harvest is low-tech and based on manual labour, techniques in the West would need to be industrialised in order to attain economic viability, so there is limited potential for adopting practices developed there to date. In terms of assessing the overall energy balance of macroalgal cultivation, the energy costs of harvesting and particularly post-harvest transport of a low energy density, bulky substrate will also need to be assessed. Frequency of harvest and integration into other facilities (offshore aquaculture, windfarms) may impact on the energy costs, but again need full assessment. Political and financial will is of critical importance in the development of pilot scale facilities that will allow these issues to be addressed. Some projects currently in their initial phase have such a focus (e.g. Energetic Algae).

Bloom: Where they occur, harvest of nuisance bloom species is a necessity if not a legal obligation (e.g. France). Currently, harvesting is carried out in order to minimise detrimental economic effects of blooms on the tourism industry and economic and environmental efficiency is not considered. Bottlenecks relate to technical challenges of harvesting good quality biomass (before degradation has begun) from on-shore/near-shore, often soft-sediment, environments, and of maximising the ratio of biomass to debris (sediment; associated biotope) harvested. In France, harvesting technology is being improved with this in mind and also in response to recently implemented changes to legislation that relate to safety of harvesting around high levels of noxious gases (H₂S). New machinery is being developed to improve both the environmental impact and the quality of the harvested algae by harvesting at an early stage of the life cycle in the wave-breaking zone (Sylvain Ballu, CEVA, personal communication).

Wild: Wild harvest of kelps is carried out to supply alginate and food/fertiliser industries in Norway and France. Techniques have been successfully adapted to local environments and conditions – e.g. the Scoubidou - for *Laminaria digitata* in France, and the cutter rake - for *Laminaria hyperborea* in Norway, reviewed in Werner and Kraan (2004). It is likely that these techniques will require adaptation to local environments in some cases but essentially the technology is in place. Besides, all easily accessible large natural seaweed resources are already being harvested (Bixler and Porse, 2010). An important unknown in terms of wild harvest is the full scale of the impact on the ecosystem, and aside from being of paramount

importance in itself, this will be likely to affect potential legislation and ultimately the possibility to harvest at all, through the granting of harvesting licences and total biomass quotas (for example see recommendations in McLaughlin et al. 2006).

2.3 Quality of the final biofuel product

At this moment aquatic biomass is not yet used in biofuel production. Before large scale biofuel production from aquatic biomass starts it is important to know whether aquatic biofuels will meet the European technical standards that are applied to biofuels. Some biofuels production processes deliver a relatively standard quality of end product, like bioethanol (European norms for bioethanol blends EN15376 and EN15376). The bioethanol molecule is the same regardless of the feedstock. For biodiesel however the quality of the oil from biomass strongly influences the quality of the biodiesel. The EU has several technical standards for biofuels also for biodiesel (for instance FAME requirements and test methods (EN 14124)) currently mostly made from rapeseed oil. These standards could be used as a benchmark to assess the suitability of aquatic biomass oil, most likely microalgal oil, for biodiesel production.

Bioethanol

Production of ethanol from an algal biomass requires the following steps:

1. growth of the starch rich biomass
2. harvesting
3. disintegration of the biomass
4. saccharification and fermentation
5. separation of ethanol

For the maximum yield of ethanol from biomass it is necessary to convert as much of the biomass as possible to sugar which is then converted into bioethanol. The sugar conversion into ethanol (fermentation) is performed by micro-organisms, the most common one being yeast. During the reaction sugar is converted into ethanol and carbon dioxide; this is also accompanied by the production of heat. The production of carbon dioxide during the conversion reaction is one of the drawbacks of bioethanol production if compared to the other biofuels due to potentially higher greenhouse gas emissions over the life-cycle. However, if the CO₂ could be used as a feed for the algal biomass growth this drawback could be overcome and the overall energetic requirement of the process could be decreased (Douskova et al. 2009).

The limitations of the biomass growth for bioethanol production are the same as for the other biofuels and are discussed above. In general, it is crucial to obtain the highest starch content in a biomass and also the highest concentration of biomass in the medium. In comparison to traditional crops (first generation biofuels) and lignocellulosic feed-stocks (second generation biofuels), algae (third generation biofuels) can provide a high-yield source of biofuels without compromising food supply chains (usage of arable land). There are algae accumulating carbohydrates (starch, cellulose), which can be used as a feedstock for the production of bioethanol. The algal starch, cellulose or other carbohydrates can be used for bioethanol production after hydrolysis.

Many of the properties of starches that determine their suitability for particular end-uses are dependent upon their amylose/amylopectin ratios. The green algal starch shows structural similarity to cereal starches (Maršálková et al., 2010). Macroalgae contain mainly cellulose that could be easily hydrolysable to

fermentable sugars (Adams et al., 2009). These features together with their growth characteristics make these photosynthetic species exploitable in large-scale processes.

Prior to fermentation the algal polysaccharides (starch, cellulose, hemicelluloses, laminarin) have to be hydrolysed to fermentable sugars by so-called saccharification (Kelsall and Lyons, 1999). The hydrolysis could be either chemical (acid hydrolysis at high temperatures) or enzymatic. The acid hydrolysis is non-specific and can lead to corrosion of equipment as well as formation of undesirable by-products decreasing the yield and/or toxic to fermenting micro-organisms. On contrary enzymatic hydrolysis is highly specific and increases the yield of starch hydrolysis (Maršálková et al., 2010).

In the next step, the fermentable sugars are converted to ethanol by a suitable yeast strain (*Saccharomyces cerevisiae*). Finally the ethanol is purified from the fermentation broth by distillation and the obtained concentrated ethanol (95%) can be blended with fossil fuels or directly used as fuel. The solid residue from the process can be used as animal feed or as a feedstock for biogas production (John et al., 2011). The limitations of the two last steps are the same which are faced during in first or second generation bioethanol production.

An alternative way to bioethanol production from algae is direct production of ethanol. This approach is overcoming the issue with algal harvesting and disintegration as well as the need for yeast since the algae directly perform the fermentation and produce ethanol (Ueda et al., 1996; Hirano et al., 1997; Ueno et al., 1998; John et al., 2011).

As stems from above the two most critical steps in ethanol production from algae are the production of the starch rich biomass and the biomass concentration. In order to cheapen the biomass production the algae can be grown on carbon dioxide derived from combustion of organic waste, fermentation processes or other sources (Doucha et al. 2005; Douskova et al. 2009; Mann et al. 2009). This characteristic enhances the ecological and economic impact of the proposed technology, because of its potential to bioremediate carbon dioxide emissions from different CO₂ sources including waste incinerators, power stations, limekilns, cogeneration units, etc. *in situ* (Nigam and Singh, 2010; Doušková et al. 2009; 2010; Kaštánek et al. 2010). The starch content of the biomass could be increased up to 50% by limitation of essential macroelements (Brányiková et al., 2010). The concentration of biomass affect the price of the harvesting, the more concentrated biomass the less energy it requires. The biomass concentration is dependent upon the bioreactor construction. However, the more productive bioreactors also tend to be more expensive to construct. Therefore a compromise between the price of the construction and production of algal biomass (productivity) must be reached for each biofuel in order to achieve maximum efficiency.

In conclusion, the greater availability of carbohydrates in algae and the possibility to process cellulosic material potentially forming residues from the use of algae for other production pathways would make bioethanol production a relatively easy production pathway requiring less technological breakthroughs than for other biofuels production pathways. This is all the more true that the larger availability of macro-algae and their easier harvestability would remove several of the bottlenecks for biofuels production usually observed when considering biofuels production.

Biodiesel

Algae produce a great variety of fatty acids and lipids which must be extracted before their conversion into fuels. The composition of microalgae lipids is qualitatively different from that of common vegetable oils and the conventional technologies for processing them may be unsuitable as they incur huge losses. In addition, the unusually high content of free fatty acids in the oil, unsaponifiable constituents, phospholipids, glycolipids and its dark colour, all cause difficulties in the necessary crude lipid refining process. Extraction of lipids from microalgae at laboratory scale has been attempted by physical methods (with a screw press) or chemically with organic solvents, water (by three-phase partitioning extraction methods) and supercritical methods; and solvent extraction has proved to be the most workable approach so far. The extraction of lipids from algae requires attention to their polarity. Polarity is related to the distribution of lipids within the algal cell and the association of lipids to non-lipid molecules. Lipids present in algae can be classified as: (i) neutral lipids (NLs) (triacylglycerols, TAGs, wax esters, hydrocarbons, fatty acids, FAs, and sterols); (ii) phospholipids (PLs) (phosphatidylcholine, PC, phosphatidylethanolamine, PEA, phosphatidylserine, PS, phosphatidylglycerol, PG, and phosphatidylinositol, PI); and (iii) glycolipids (GLs) (sulfoquinovosyldiacylglycerol, SQDG, monogalactosyldiacylglycerol, MGDG, and digalactosyldiacylglycerol, DGDG) (Pohl and Zurheide, 1982). TAGs are usually regarded as energy storage products, whereas PLs and GLs are structural lipids, contained mainly in the cell membranes.

Typically, chain lengths of over 16 carbons are found in freshwater microalgae whereas in marine microalgae chain lengths of 18 carbons are found and the presence of polyunsaturated fatty acids (PUFAs) is also usual, the main ones being eicosapentaenoic acid (EPA, C20:5n3), and docosahexaenoic acid (DHA, C22:6n3). These fatty acids are susceptible to oxidation during storage, which makes them less suitable for use in biodiesel. The final result of autoxidation is the transformation of lipids into some other deteriorated lipid macromolecules that do not meet the legal specifications for using as biodiesel (Porter et al., 1995).

Also, the microalgae species and the time of harvesting will determine the lipid composition of algae. Algae harvested in the exponential growth phase will contain more polar lipids (GLs and PLs) than those harvested in the late stationary phase of growth, which contain more neutral lipids (TAGs). The lipid fractionation procedure and the selection of solvent, or solvent system, will depend on the particular classes of lipids present. Overall, neutral lipids are recovered by non-polar solvents, whereas polar lipids are recovered by polar ones. Therefore, to select a solvent system we need to match the polarity index and the solubility parameters of the solvents and the lipids classes to be extracted.

2.3.2.1 Biomass pretreatment

Lipids from microalgae may often be extracted in the wet state directly after harvesting. The cells do not need to be homogenized since they are readily broken on suspension in the extracting solvent. In some cases, breakage of the cell wall may be necessary to: (1) reduce the extraction time, (2) avoid the use of high temperatures and pressures to push the solvent into contact with the lipids within the cell, (3) reduce solvent consumption, (4) allow the solvent to easily penetrate into the cell and release cell contents into the bulk medium for increasing the lipid yield. This may be accomplished by using one of the well established cell-disruption techniques such as sonication, homogenization in a tissue grinder, blender or in high-pressure flow device, freezing and grinding with a pestle and mortar, autoclaving microwaves and osmotic shock (Chisti and Moo-Young 1986, Garrido et al., 1994). For large-scale cell disruption for the disintegration of algae, the mechanical method of shearing the cells with a bead mill appears more suitable. Cerón et al., (2008) demonstrated that disruption was necessary for the algae *Scenedesmus almeriensis*.

This work compares three different methods: mortar, bead mill, and ultrasound. The best option among, bearing in mind their potential industrial application, proved to be the bead mill with alumina in a 1:1 w/w proportion as disintegrating agent for 5 min. In general bead mill may be the pre-treatment choice for microalgae cells with strong cell wall.

Within the mechanical disruption methods, homogenizers (pumping of microalgae slurry through a restricted orifice valve) such as French press, are less time consuming than other methods, easy to use and can ensure almost complete breakage of small cells. It is recommended for microalgae with the strongest cell wall which require up to 600 bar. Cooney et al., (2009) have explored many chemical (dissolution in 1M NaOH(aq), hydrogen peroxide treatment, or adding detergents) and physical techniques (sonication, glass bead beating, liquid nitrogen + grinding the frozen mixture) applied to wet and dried *Nannochloropsis* and *Tetraselmis* biomass. Except for the grinding of cells in liquid nitrogen, none of the physical and chemical methods tested were effective in cell disruption.

The same authors have tested the suitability of grinding cell after oven drying and compared it with wet cell that had been ground and not ground. The solvent system used extracting lipid was chloroform:methanol:water (1:2:0.8 v/v/v). The best lipid recovery yield was achieved from solvent applied to oven dried and ground cells (21.5%), but the extraction applied to wet cells that had been ground was 20%, suggesting that the presence of water has the effect of blocking solvent access. Recently Lee et al., (2010) compare several methods for effective lipid extraction from three different microalgae: *Botryococcus sp.*, *Chorella vulgaris*, and *Scenedesmus sp.* Among the five methods used (autoclaving, bead mills, microwave, sonication, and osmotic shock with a 10% NaCl solution), the microwave oven method (at about 100 °C and 2450 MHz for 5 min) appears to be the most simple, easiest and most efficient method for lipid extraction from microalgae.

2.3.2.2 Extraction of lipids from microalgae

For complete extraction, all the linkages between the lipids and other non-polar lipids cell components must be disrupted and, at the same time, the disruption agent used must not cause any degradation of the lipid extracted. To carry out the extraction in large quantities, an extraction tank with a reflux apparatus, equipped with cell homogenizing apparatus such as polytron homogenizer, may be employed. Usually, the non-polar and the polar solvents are mixed in a ratio of the former to the latter ranging from approximately 2:1 to 1:1 by volume. Solvents can be chloroform, methanol, isopropanol and others although biocompatible solvents as ethanol and hexane are preferred. The extraction may be carried out at 60 °C by using from 5 to 20 parts by volume of the mixed solvent per part by volume of dry biomass. Although there is no limit, the extraction period usually ranges from 30 min to 3 h. After extraction, the solvent may be removed by distillation under reduced pressure at temperatures ranging from 40-60 °C to provide a lipid composition that contains TAGs, PLs, GLs, glycol-phospholipids, chlorophylls, beta-carotene, sterols, etc.

Once the lipids have been extracted, the crude extract obtained must be purified before commencing the conversion of lipids to methyl esters of fatty acids. The usual laboratory scale methods for purifying lipids are based on the difference in affinity of the polar lipids and their contaminants for a certain solvent. The crude extract is treated with non-polar solvents such as chloroform, hexane or diethyl ether, in which the non-lipid contaminants are less soluble (Molina Grima et al., 1994). These procedures do not attain the complete extraction of most polar lipids (e.g., proteolipids) because of their low solubility in these solvents. Processes for refining of crude algal lipid extracts (mainly the removal of the phospholipids fraction), similar

to those existing for terrestrial plants (Nasirullah, 2005), have not been developed to date. Unlike terrestrial feedstocks, the unusual content of microalgal oils in fatty acids, non-saponifiable constituents, PLs, GLs and its dark colour, may all cause difficulties in the refining processes. An alternative to the refining may be the lipid fractionation and to properly process each lipid classes separately.

2.3.2.3 Fatty acid extraction by direct saponification

Direct saponification of microalgae biomass enables fatty acids to be obtained as potassium or sodium salts instead of as crude lipids in a first step. The direct extraction/saponification is performed a room temperature for 8 h or at 60 °C for 1 h. After saponification, the unsaponifiable fraction can be extracted with hexane and then, the hydroalcoholic phase, containing the fatty acid salts, is acidified by HCl addition to pH 1, and the fatty acids obtained are recovered with hexane. Such direct saponification during extraction from the biomass is faster, and reduces cost and operating time compared to lipid extraction followed by saponification, although more intensive operating conditions are necessary (1 h at 60 °C or 8 h at room temperature). The resulting fatty acid extraction yields are somewhat lower than for lipid extraction with the same solvent system and the order of efficiency is similar to that obtained for lipid extraction. It may therefore be assumed that direct saponification of biomass could ideally be induced in two stages, first lipid extraction with a solvent, and second alkaline hydrolysis of the extracted lipids to render fatty acid salts. The yields obtained from fatty acid extraction carried out in this way would depend mainly on the suitability of the extraction solvent (Ibañez-González, 1998).

2.3.2.4 Direct transesterification of microalgae biomass

Direct transesterification of microalgae biomass, without previous separation of lipids, is a relatively low expense process for recovering and fractionation of fatty acids methyl esters from microalgae. The process was developed at the University of Almería, Spain, by Belarbi et al., (2000). All aspects of the process have been demonstrated at lab scale. The process was initially developed for the production of high grade polyunsaturated fatty acid methyl esters. The direct production of FAMES uses either wet or dry biomass. In a typical run, biomass paste was added to a mixture of methanol and catalyst (acetyl chloride or sulfuric acid). The resulting slurry was placed in a stainless steel pressure vessel and kept at 100°C for 120 min from the time the pressure reached its maximum value of 2.5 bar. After cooling to ambient temperature, hexane was added to the slurry reaction mixture, next the hexánica FAMES solution was removed by centrifugation. The crude FAMES extract is further polished by filtration and the hexane is finally recovered by evaporation. Because the method is not able to remove all the pigment in the crude extract, a final polishing distillation of FAMES is still needed to improve the colour of the final product. The FAMES recovery yield ranged from 90-92% (Molina et al., 1999).

Lewis et al., (2000) have studied the extraction of lipids from freeze-dried biomass of two lipid-producing microheterotrophs by two procedures: (i) the extraction of lipids from biomass by the Bligh and Dyer method followed by the transesterification of fatty acids (extraction-transesterification); and (ii) the direct transesterification of biomass to produce fatty acid methyl ester (i.e. without the initial extraction step). They demonstrated that direct transesterification of biomass was most efficient than that of the most efficient method for extraction of fatty acids prior transesterification. Recently this direct acid-catalyzed biomass transesterification has also been proven to be a promising technology for the production of biodiesel from feedstock that contains high amounts of free fatty acids such as *Jatropha curcas* seed oil

(Shuit et al., 2010). The yield of FAME extraction obtained with this seed oil was even greater (99.8%) than the one obtained for wet microalgae biomass (92%) and even greater than the traditional two step process followed for oil from oleaginous plants: extraction of the oils followed by transesterification. Therefore, acid-catalyzed direct transesterification of biomass can be an important technology for biodiesel production not only for microalgal oils.

3 Downstream by products - Value added algae biofuel byproducts

3.1 Microalgae

The vast biodiversity of microalgae results in the production of an enormous variety of microalgal metabolites, of which the full potential is yet to be explored. This biodiversity and the recent developments in genetic engineering, make microalgae one of the most promising sources for new products and applications. A case study shows the necessity of biorefinery and usage of high value byproducts by with example of biodiesel production of microalgae. This case study will be followed by a range of examples of promising applications of aquatic biomass. In recent years this exploration has resulted in very promising applications in the field of production of oils and fatty acids, fluorescent pigments, stable-isotope biochemicals, bioactive compounds (Apt & Behrens, 1999; Pulz & Gross, 2004; Spolaore *et al.*, 2006), pollution abatement (Wang *et al.*, 2008; Bozarth *et al.*, 2009), diatom nanotechnology (Lopez *et al.*, 2005; Bozarth *et al.*, 2009) and renewable energy (Li *et al.*, 2008; Skjanes *et al.*, 2007).

Owing to their global composition, microalgae are generally used in the field of human and animal nutrition, although only a few species are currently allowed for human nutrition. However, the possibility of growing their high value components, e.g. fatty acids, in sufficiently high concentrations under controlled circumstances, has allowed the commercialization of these high value products (Radmer, 1996; Apt & Behrens, 1999; Spolaore *et al.*, 2006). If the current costs of microalgae production could be sufficiently reduced, CO₂ biofixation would be possible on a large scale, and the markets of low value products, like novel industrial materials and renewable energy, would open up. In the following paragraphs the current and future applications of microalgae are highlighted.

Necessity of value bioproducts: microalgal biodiesel case

Value added byproducts from algae will be from importance if biodiesel production has to be economical feasible. To prove this the question is raised whether it is economically feasible to produce biodiesel from microalgae if the cost price of biomass production can be reduced to 0.40 €/kg. If we assume that algae contain 40% lipids and the value of biodiesel is 0.50 €/liter, the value of the biomass used for biodiesel production is only 0.20 € /kg. It also needs to be considered that costs for extraction of the lipid and conversion of the lipids into biodiesel were not even taken into account. This means that it will not be feasible to produce algae for the production of biodiesel only (Norsker *et al.* 2010).

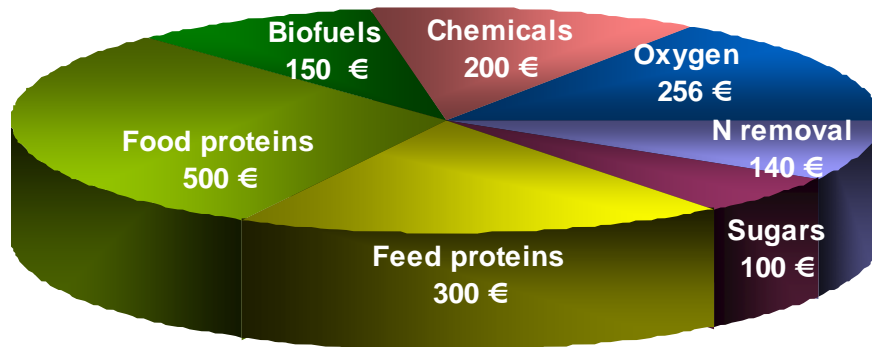


Figure 3: Value of algal biomass per 1,000 kg after biorefinery (Norsker et al. 2010).

For this reason it is necessary to look at the possibility to refine algal biomass into different products and analyze the total value of the biomass. For this combinations of high-value products in niche markets are not assumed because the market volumes of high-value products and biodiesel are incompatible. In the study of Norsker *et al.* (2010) the assumption was made that biorefinery of algal biomass into products for bulk markets make use of the functionality of the products. The case is randomly chosen and is only used to analyze whether the total value of the biomass produced is sufficient to allow production costs of 0.40 €/kg (Figure 3) (Norsker *et al.* 2010).

During this study it was assumed to produce algal biomass with consisting out of 40% lipids, 50% proteins and 10% of carbohydrates. (Norsker *et al.* 2010) A more detailed overview is presented in Carioca *et al.* (2009). When these different fractions are separately extracted via biorefinery the biomass gains additional value:

If the lipid fraction is not only used for production of biodiesel but also as a feedstock for the chemical industry (e.g. in coatings) or for edible oils (e.g. ω -3-fatty acids) the lipid fraction of the algae increases in value. In this case it was assumed that 25% of the lipids are used for these functional products with an estimated value of 2 €/kg and 75% is used for biodiesel production with a value of 0.50 €/kg.

Next to that proteins could be fractionated as well into a water soluble fraction (e.g. Rubisco) of 20% of the proteins and a water insoluble fraction (80%) taken into account that the water soluble fraction has food value (5 €/kg) and the insoluble fraction a feed value (0.75 €/kg).

Finally a carbohydrate fraction of 10% was assumed. The carbohydrates in algae are very low in cellulose. They are in general storage products as fructans, glucans and glycerol which can be used as chemical building blocks or for production of bio-energy. We assumed the value of carbohydrates to be 1 €/kg. Besides these main products, there are additional by products such as reduction of nutrients in waste streams and production of oxygen.

In wastewater treatment removal of nitrogen compounds via nitrification and denitrification is an expensive process; the cost of nitrogen removal is 2 €/kg. Microalgae contain 70 kg of nitrogen per 1000 kg of microalgae. If algal production would be combined with wastewater treatment we would save 140 € for nitrification and denitrification per ton of algae produced. A similar analysis could be made for phosphate. Algae produce oxygen rich gas. Per ton of algae 1,600 kg of oxygen rich gas is produced. In aquaculture oxygen rich gas is used for supply of sufficient oxygen to the fish. The value of the gas produced is approximately 0.16 €/kg of oxygen.

If the total value of all these products is added up we come to a total value of the biomass of 1.65 €/kg of algae. Of course the refinery of these components will be at a certain cost. The analysis shows, however, that in case algal biorefinery is used the total value is higher (1.65 €/kg) than the total cost for algae production (0.40 €/kg) and makes it worthwhile to develop this approach.

Overall it was concluded that only production of biodiesel from microalgae is economically not feasible but that an integrated biorefinery concept of microalgae with biodiesel as one of the products can lead to a feasible process.

- Algal biomass

Traditionally algae biomass is used in food and feed applications as a dietary supplement (Spolaore *et al.*, 2006) or in the cosmetic industry. Although algae produce high quality protein, essential fatty acids and vitamins, their high production costs as well as technical difficulties to incorporate the algal material into palatable food preparations, have limited their application in food and feed to nutritional supplements and a source of natural food colorants (Becker, 2007; Spolaore *et al.*, 2006). The microalgal biomass market has a size of about 5000t/year. Microalgal biomass, used in health food, functional food and animal feeds, represents the most important economic application of microalgal biotechnology (turnover of ca. U.S.\$ 1.25x10⁹/year) (Pulz & Gross, 2004). Microalgae used as dietary supplements have demonstrated various health-promoting effects, e.g. antitumor action and improved immune response (Liang *et al.*, 2004; Becker, 2004). Algae are indispensable in aquaculture as they are the natural source of PUFA's for these animals. They are used as sole component or as food additive to supply basic nutrients, color the flesh of e.g. salmonids or for inducing other biological activities (Muller-Feuga, 2000). It is estimated that about 30% of the current world algal production is sold for animal feed application (Becker, 2007).

- Fatty acids

The average lipid content of algal cells varies between 1% and 70%, but can reach 90% of their dry weight under certain conditions. For a number of algal groups, this lipid fraction is very rich in ω 3- and ω 6-long chain polyunsaturated fatty acids (LCPUFA's). Because LCPUFA's are essential for the functioning of higher organisms and higher organisms cannot produce them *de novo*, LCPUFA's have to be available in their diets. Although fish and fish oils are considered a good source of LCPUFA's, toxin contamination problems in the concentrated fish oils have raised interest in microalgal LCPUFA's (Apt & Behrens, 1999; Spolaore *et al.*, 2006). The LCPUFA's of particular interest are docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), arachidonic acid (AA) and γ -linolenic acid (GLA). At present, only heterotrophically produced microalgal DHA is commercially available. Commercial applications include the DHA-enrichment of vegetarian oil, eggs and commercial formulas for children (Spolaore *et al.*, 2006). EPA can only be accumulated in high

concentrations by obligate phototrophs, which results in very high production costs and limited commercial use (Ward & Singh, 2005).

Because algae are capable of synthesizing LCPUFA's, algae could serve as a source of genes involved in LCPUFA synthesis. Other organisms; like higher plants or yeasts, could be engineered to produce LCPUFA's as a dietary source of these fatty acids (Ward & Singh, 2005). Big companies, such as DSM/Martek is currently a very strong player in the microalgae field: they have a lot of strong patents and a very strong commercial position. Getting a foothold in this market is therefore a challenge.

Pigments

Two groups of microalgal pigments have commercial applications, i.e. the carotenoids and the phycobiliproteins. Carotenoids are mainly used as natural food colorants (e.g. orange juice) and as additive for animal feed (poultry, fish). They have also applications in cosmetics. The nutritional and therapeutic relevance of certain carotenoids is due their ability to act as provitamin A, that is, they can be converted into vitamin A (Spolaore *et al.*, 2006). Assimilation of carotenoids contained into the biomass is a function of their overall composition, as fiber and lipids content (Granado-Lorencio *et al.*, 2009). The worldwide market of all commercially used carotenoids is estimated at US\$ 887 million for 2004 (Del Campo *et al.*, 2007).

Another important group of pigments are the phycobiliproteins. Their ability to form stable conjugates with many materials, e.g. antibodies, and their excellent absorption properties, allows them to function as highly sensitive fluorescent tags for labeling highly specific probes to identify cell types or proteins, and to be applied among other things in flow cytometry and immune-histochemistry (Apt & Behrens, 1999). Phycobiliproteins can be recovered by novel technologies including expanded bed chromatography, thus allowing a wider availability of these compounds for other purposes (Bermejo *et al.*, 2007). Thus it are also used as food pigments, replacing synthetic pigments, and as pigment for natural cosmetics. Their global market was estimated at more than US\$ 50 million in 1997 (Spolaore *et al.*, 2006).

Cosmetics

Extracts of several microalgal species have been used in commercial products in the skin care market, and some cosmeticians have even invested in their own microalgal production system, however it may be difficult to combine cosmetic products with biofuel production : cosmetic industry wants now very neutral products. Microalgal fatty acids processed in lipid-based cosmetics are gaining commercial importance because of their provision of nourishing and protecting effects to the skin (prevention of scaly dermatitis and skin dehydration) (Kim *et al.*, 2008; Pulz & Gross, 2004). Tocopherol and mycosporine-like amino acids isolated from certain microalgae species protect the skin against UV radiation, and are therefore applied as sunscreens in cosmetics. Other active ingredients are used in cosmeceuticals which prevent blemishes, repair damaged skin and inhibit the inflammation process. Microalgal extracts have also shown to stimulate collagen synthesis in the skin, leading to their use in products supporting tissue regeneration and wrinkle reduction (Kim *et al.*, 2008). As mentioned above, microalgal carotenoids and phycobiliproteins also serve as natural colorants in cosmetics (Kim *et al.*, 2008).

Isotopically labeled compounds

Microalgae are ideally suited as sources of stable isotopically labeled compounds. They are easily handled and cultured, and their ability to perform photosynthesis allows them to incorporate ^{13}C , ^{15}N and ^2H from relatively inexpensive inorganic compounds (i.e. $^{13}\text{CO}_2$, $^{15}\text{NO}_3$ and $^2\text{H}_2\text{O}$) into more highly valued organic compounds (Acién *et al.*, 2003). These isotopically labeled compounds are incorporated in culture media of e.g. mammalian cells, and are subsequently incorporated into cellular components such as proteins. This technique can be used to determine molecular structure, study molecular interaction, elucidate metabolic pathways (Apt & Behrens, 1999) and for clinical diagnosis tests (Radmer, 1996). The market of stable isotopically labeled compounds is probably higher than US\$ 13 million/year (Spolaore *et al.*, 2006).

Bioactive compounds

A large number of bioactivities have been reported in algae, including anticancer, antimicrobial, anti-HIV, antiviral and various neurological activities. None of those compounds have yet become a commercially useful pharmaceutical (Kirk & Behrens, 1999; Pulz & Gross, 2004). So far, microalgae have not been as intensively screened for pharmaceutically interesting products as other microorganisms and higher plants. Because the probability of developing a successful pharmaceutical directly correlates with the amount of screening that is done, the lack of a commercial product could be due to the relatively limited amount of screening of algal samples (Kirk & Behrens, 1999).

Nanotechnology

Diatoms biomineralize a mixture of silica, proteins, and carbohydrates to form an intricately patterned inorganic silica shell that surpasses modern engineering capabilities. These frustules serve as a biomimetic model silica structures at the micro-, meso-, and nanoscale; and are presently commonly used as diatomaceous filters in applications ranging from filtering liquids to DNA purification to adsorbing heavy metals (Bozarth *et al.*, 2009). There are ca. 250 living diatom genera with more than 200 000 estimated species classified by their unique morphologies (Gordon *et al.*, 2008). The ability to genetically engineer diatoms to synthesize designer frustules is anticipated to have wide range applications as microelectronic devices, chemical and biological sensing and diagnosis, as drug delivery systems, catalysis, highly efficient nanofiltration, and energy storage. Extensive fundamental research will be needed however, to unravel the genetic and molecular components as well as the mechanisms involved in diatom silica frustules synthesis (Bozarth *et al.*, 2009).

Novel industrial materials

Algal biomass can be incorporated in construction materials, like plastics. Using natural fibers to reinforce plastics reduces the weight and cost of those plastics and is environmentally advantageous. This fast growing market is currently dominated by the use of wood followed by agricultural by-products. Whole algae cells can be directly incorporated in plastics, and composite materials have successfully been constructed from algae and polypropylene, polyethylene and PVC. Algal biomass can be incorporated up to 50 wt%, and these materials have a large variety of uses (Skjanes *et al.*, 2007).

- Pollution abatement

Algae provide a means for CO₂ biofixation through their photosynthesis activity. Moreover, the production of microalgae biomass at large scale requires the direct utilization of flue gases instead of pure CO₂ as carbon source. As algae require large amounts of nutrients, it can be advantageous to combine CO₂ fixation from gaseous streams (i.e. combustion systems) with wastewater treatment. This could also help removing hazardous combustion products such as NO_x and SO_x. To make this process economically realistic, productivities near the theoretical maximum, high-energy prices, and greenhouse gas abatement credits would be required (Muñoz & Guieysse, 2006). However, CO₂ biofixation with closed material cycles combining different effluent fluxes with value-added algae and algae derivatives production, could be economically feasible (Pulz & Gross, 2004; Wang *et al.*, 2008).

Next to the removal of nutrients from wastewater, algae can be used for metal removal from wastewater through intracellular and extracellular metal chelation by phytochelatin. Phytochelatin are chelators produced by algae upon exposure to heavy metals. Through this chelation mechanism heavy metals are detoxified both within cells and tissues as well as in the extracellular space. Understanding the mechanistic details of chelator-substrate binding and the (over)expression in algae could lead to applications in the collection of metal contaminants in polluted waters and in phytoremediation of contaminated sites by algae (Vílchez *et al.*, 1997; Bozarth *et al.*, 2009).

- Proteins

Shortage of protein foods in various areas of the world is of increasing concern. Because of overpopulation and a scarcity of land for production of sufficient protein at a cost within the reach of the people, means of producing protein-rich foods in areas of shortage are being sought. (Bessie B. Cook 1962).

Spirulina (Cyanobacteria) is well-known since centuries to be a good food source : people living near lac Tchad harvest it naturally from inside, dry it and use it as a main source of food. Nowadays, Spirulina is industrially grown in raceways all over the world due to its high content of protein (almost 70%) (Tolga G.KSAN, Ayβeg.l ZEKERÜYAOÚLU, Ülkür AK 2006). Spirulina can also be used for feed applications. An experiment shows that it will be a very good source for shrimp production (R. Hanel*, D. Broekman, S. de Graaf and D. Schnack 2007).

Spirulina is not the only source of microalgal proteins. A study shows that in 4 different species (Tetraselmis, Dunaliella, Chlorella and Isochrysis), the protein content ranged from 39% to 54% on DM. Amino acid profile is very comparable to the FAO standard : Microalgae can be considered as a very good source for Single Cell Protein (Fabregas, J.; Herrero, C. 1986)

Production of protein from biodiesel microalgae source can be very interesting in the field of feed. A huge quantity of byproduct biomass rich in protein will be produced. Feed industry is seeking new sources of proteins in the field of aquaculture for example.

Proteins can also be hydrolyzed to generate high value bioactive peptides. A study shows for example that Chlorella pretien can be hydrolyzed with pancreatin to generate an immune-active peptide. (Humberto J Morris *et al.* 2009)

Enzymes

As any living cell, microalgae possess a huge number of enzymes, both inhibitory and activatory that can be useful for industry.

Microalgae can synthesize starch. Alpha-amylase, Beta-amylase or branching enzyme are an area of interest for starch industry. Microalgae can be found in a very diversified biotope, we can expect found extreme conditions enzymes.

A study shown that *Porphyridum Cruentum* is a good source of Super Oxyde Dismutase (SOD). This enzyme can be used. This enzyme can be used by industry for antioxidant supplement. (Misra and al. 1977).

Peptides from microalgae can also be used for pharmaceuticals by acting on body enzyme.

3.2 Macroalgae

Macroalgae have been reported to be a large reservoir of molecules, several of these molecules are important to the industry (both in terms of volume and economical value), as is the case for phycocolloids (Alginates, Agar and Carrageenan total sales reaches 1,018 M US\$ in 2009, Bixler and Porse, 2010) whereas some are more recently described as novel bioactive compounds and represent limited volumes (e.g. halogenated furanones from *Delisea pulchra* or Kahalalide F from a specie of *Bryopsis* as a possible treatment for lung cancer, tumors and AIDS). Not all new biodiscovery from macroalgae are compatible with biofuels production, and the Biofuel downstream by-products from macroalgae will be discussed in this document.

Metabolites from macroalgae

The diversity of metabolites found in macroalgae now, are reflecting both their place in evolution and the adaptation to a moving and highly variable habitat, i.e. the intertidal zone, a mix of dynamical constraints (wave and wind action, mechanical sweeping of the surrounding seaweeds), desiccation (exposition to UV radiation, wind, salt concentration, etc.), and competitive ground for settlement, light availability and nutrient to name but a few factors. Modern screening programmes for drug development are combined with ecological observation and includes specimens with unique (usually chemical) mechanisms for coping with environmental pressures (Haefner, 2003).

Some of the metabolites are currently used in different industry sectors. The non-exhaustive list below sums up the various species and their application in some industrial sectors. The specie names are listed as they appeared in the literature; recent updates in the taxonomy might have affected some of the specie names (please refer to the recent taxonomical publications or electronic publication by Guiry & Guiry (2011) for recent updates).

| Sector | Applications | Specie |
|---------------------------------|---|---|
| Human consumption (Europe only) | Sea vegetables, food ingredients | <i>Saccharina latissima</i> , <i>Laminaria hyperborea</i> , <i>Undaria pinnatifida</i> (Wakame), <i>Fucus serratus</i> , <i>Fucus vesiculosus</i> , <i>Fucus spiralis</i> , <i>Ascophyllum nodosum</i> , <i>Himanthalia elongata</i> , <i>Alaria esculenta</i> , <i>Porphyra yesoensis</i> (Nori), <i>Porphyra tenera</i> (Nori), <i>Porphyra leucostica</i> , <i>Porphyra linearis</i> , <i>Porphyra umbilicalis</i> , <i>Palmaria palmata</i> , <i>Chondrus crispus</i> , <i>Mastocarpus stellatus</i> , <i>Iridaea edulis</i> , <i>Enteromorpha prolifera</i> (Ao Nori), <i>Enteromorpha linza</i> (Ao Nori), <i>Ulva rigida</i> |
| Animal feed | Food additive Trace element and vitamins source Protein source for fish, shellfish, poultry, cattle Animal feed supplement Antibiotics against fish pathogens Protein source for fish, shellfish | <i>Alaria esculenta</i> <i>Ascophyllum nodosum</i> <i>Palmaria palmata</i> <i>Phymatolithon calcareum</i> , <i>Lithothamnion corallioides</i> (Maerl) Red algae (unspecified) <i>Ulva spp.</i> |

| | | |
|---------------|---|---|
| | poultry, cattle | |
| Health | Nutraceuticals (Functional food, food supplements) | <i>Alaria esculenta, Saccharina latissima</i> |
| | Seaweed bath, wrap, spa, thalassotherapy | <i>Ascophyllum nodosum, Fucus spp.</i> |
| | Calcium supplements (aragonite) | <i>Padina pavonica</i> |
| | Para-pharmaceuticals | <i>Palmaria palmata Porphyra spp., Chondrus crispus</i> |
| Cosmetics | Seaweed extracts as additives with claimed function (Antioxidant, moisturising, antibacterial, anti-UV) | <i>Himanthalia elongata</i> |
| | Emulsifier and emulsion stabilizer in cream and lotions. (alginate/carrageenan) | <i>Laminaria digitata, Laminaria hyperborea, Saccharina japonica, Macrocystis pyrifera, Ascophyllum nodosum, Ecklonia maxima., Gigartinaeae, Solieriaceae, Phylloporaceae, Hypneaceae</i> |
| | Excipient absorbable in gels, creams, ointments and pomades. (alginate/carrageenan) | <i>Laminaria digitata, Laminaria hyperborea, Saccharina japonica, Macrocystis pyrifera, Ascophyllum nodosum, Ecklonia maxima., Gigartinaeae, Solieriaceae, Phylloporaceae, Hypneaceae</i> |
| | Seaweed extracts as additives with claimed function (anti-cancer, anti-UV, nutritive) | <i>Palmaria palmata</i> |
| | Foam stabilizer (Alginate/carrageenan) | <i>Laminaria digitata, Laminaria hyperborea, Saccharina japonica, Macrocystis pyrifera, Ascophyllum nodosum, Ecklonia maxima., Gigartinaeae, Solieriaceae, Phylloporaceae, Hypneaceae</i> |
| | Seaweed extracts as additives with claimed function | <i>Chondrus crispus</i> |
| | "Peeling" effect in creams | <i>Lithothamnium calcareum</i> |
| Agrochemicals | Bioactive algal compounds with proven effects (e.g. Anti-wrinkle, anti-acne, anti-dandruff), "phykosyl" | <i>Asparagopsis armata</i> |
| | Growth enhancer | <i>Laminaria digitata</i> |
| | Stimulant for plant defence systems ("Plant vaccine"), liquid extracts. | <i>Ascophyllum nodosum, Fucus spp., Ecklonia maxima, Laminaria spp., Durvillaea spp.</i> |
| | Fungicides | <i>Dictyopteris undulata, Dictyopteris zonaroides, Dictyopteris justii, Chondria spp., Laurencia spp., Asparagopsis armata, Falkenbergia spp.</i> |
| Soil additive | <i>Phymatolithon calcareum, Lithothamnion corallioides (Maerl)</i> | |
| Bactericides | Red algae | |
| Biotechnology | Surface protecting substances (alginate) | <i>laminaria spp.</i> |
| | Enzymes for specifically modifying chemical compounds | <i>Fucus spp.</i> |
| | Biomedicine (surgery, transplantation, encapsulation, etc) | Brown algae (alginate) |
| | Immobilization of cells, enzymes on gel (alginate/carrageenan/agar) | <i>Laminaria digitata, Laminaria hyperborea, Saccharina japonica, Macrocystis pyrifera, Ascophyllum nodosum, Ecklonia maxima. Gelidium spp., Pterocladia spp.,</i> |

Gracilaria spp., Gigartinaceae, Solieriaceae,
Phyllophoraceae, Hypneaceae

Food engineering Red algae

| | | |
|---|--|--|
| Biomedecine | Bioactive algal compounds with activities, such as: | |
| | Antiviral (neoagarobiose HP) | <i>Gelidium spp.</i> |
| | Antiviral (carrageenan iota and lambda) | <i>Chondrus crispus</i> |
| | Antiviral (sulfoglucine) | <i>Palmaria palmata, Dumontiaceae</i> |
| | Antiviral (cetonic extracts) | <i>Cladophora rupestris</i> |
| | Antibacterial (furanone) | <i>Pelvetia canaliculata, Polysiphonia nigrescens, Iridaea violacea, Delisea pulchra, Spongomorpha elonga</i> <i>Laminaria digitata, Chondria littoralis, Cystoseira spp., Sargassum spp., Dictyota spp., Delisea fimbriata, Ceramium rubrum, Chondria littoralis, Laurencia hybrida, Laurencia ridea, Ulva lactuca, Caulerpa taxifolia, ,Caulerpa proliera, Udotea flabellum, Codium ramosum, Cladophora rupestris, Derbesia enita, Caulerpa lentillifera, Asparagopsis armata, Asparagopsis taxiformis,</i> |
| | Antibacterial (terpenes) | <i>Falkenbergia hildenbrandii, Bonnemaisonia hamifera, Gracilaria gracilis, Hypnea musciformis, Anadyomene stellata</i> |
| | Antibacterial (Halogenated aliphatics) | <i>Dictyopteris zonaroides, Dictyopteris justii, Chondria californica</i> |
| | Antibacterial (cyclical polysulfides) | <i>Fucales, Dictyotales, Sphacelariales, Laminariales Halopteris scoparia, Halopteris incurvus, Delesseria sanguinea, Rhodomela confervoides, Polysiphonia nigrescens, Polysiphonia urceolata, Polysiphonia lanosa, Dasya pedicellata, Caulerpa lentillifera, Caulerpa racemosa, Bryopsis plumosa, Cladophora cornuta, Acetabularia fragilis</i> |
| | Antibacterial (phenolic compounds) | <i>Desmarestia aculeata</i> |
| | Antibacterial (sulfuric acid) | <i>Dictyopteris undulata</i> |
| | Antifungal (hydroquinone) | <i>Dictyopteris zonaroides, Dictyopteris justii</i> |
| | Antifungal (sterols isozonanol and zonanol) | <i>Chondria spp., Laurencia spp., Asparagopsis armata, Falkenbergia spp.</i> |
| | Antifungal | |
| Antihelminthic (Kainic and allokainic acid) | <i>Digenea simplex</i> | |
| Antihelminthic (domoic acid) | <i>Chondria armata</i> | |
| Antihelminthic (active compounds not defined) | <i>Durvillaea antarctica, Sargassum vulgare, Coralina officinalis, Palmaria palmata, Hypnea musciformis, Ulva lactuca</i> | |
| Hypocholesterolemic | <i>Sargassum muticum, Fucus serratus, Pelvetia spp., Laminaria spp., Undaria pinnatifida, Chordaria spp., Heterochordaria spp., Gelidium latifolium, Porphyra spp., Gelidium amansii, Enteromorpha compressa, Enteromorpha prolifera, Ulva pertusa, Monostroma nitidum</i> | |

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| Hypotenser | | <i>Laminaria digitata, Saccharina latissima, Saccharina japonica, Undaria pinnatifida, Undaria undaroides, Ecklonia cava</i> |
| Anti-coagulant | | <i>Laminaria spp., Ecklonia spp., Pelvetia spp., Fucus spp., Ascophyllum nodosum, Delesseria spp., Asparagopsis spp., Halopteris spp., Chondrus spp., Euchema spp., Gigartina spp.</i> |
| Anti-mitotic | | <i>Fucus vesiculosus, Ascophyllum nodosum, Padina pavonica, Spatoglossum schmittii, Stypopodium zonale, Laurencia pinnatifida, Laurencia hybrida, Ceramium rubrum, Asparagopsis spp., Hypnea spp., Spyridia spp.</i> |
| Anti-inflammatory | | <i>Ascophyllum nodosum</i> |
| Neuroactive (caulerpicin) | | <i>Caulerpa spp.</i> |
| Neuroactive (costadiol, anxiolytic) | | <i>Plocamium costatum</i> |
| Neuroactive (deridol, enhance effect of phenobarbital) | | <i>Laurencia derida,</i> |
| Insecticide and anti-epidemic (phenolic + halogenated compounds) | | <i>Laurencia pinnatifida, Laurencia obtusa, Odonthalia flacosa, Plocamium cartilagineum</i> |
| Bioconversion of seaweeds (Mostly research trials) | Biomethane/Biogas | <i>Alaria esculenta, Laminaria hyperborea, Ascophyllum nodosum, Saccorhiza polyschides, Saccharina latissima, Saccharina japonica, Sargassum spp, Fucus spp., Chondrus crispus, Gracilaria tikvahiae, Gracilaria verrucosa, Iridaea spp. Ulva spp., Codium fragile, Chaetomorpha aerea, Cladophora spp..</i> |
| | Bioethanol | <i>Laminaria digitata, Laminaria hyperborea, Saccharina latissima, Saccharina japonica, Macrocystis pyrifera.</i> |
| Environmental uses | Aquaculture nutrient output purification | <i>Ulva spp.</i> |
| | Heavy metal chelation | <i>laminaria spp.</i> |
| | Carbon sink? | <i>Saccharina japonica</i> |
| | Integrated Multitrophic Aquaculture in tanks | <i>Saccharina latissima, Gracilaria lemaneiformis, Gracilaria bursa pastoris, Gracilaria chilensis, Gracilaria tenuistipitata, Gracilaria conferta, Gracilariopsis longissima, Gracilaria sp., Palmaria mollis, Palmaria palmata, Chondrus crispus, Ulva rigida, Ulva rotundata, Ulva lactuca, Ulva sp.</i> |
| | Integrated Multitrophic Aquaculture at sea | <i>Macrocystis pyrifera, Saccharina latissima, Laminaria digitata, Laminaria hyperborea, Saccorhiza polyschides, Gracilaria chilensis, Palmaria palmata</i> |

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3.2.1.1 Prices of macroalgal metabolites

As for all prices, they are subject to variations, depending on the quality and quantity of the products (origin, purification grade, etc.). It must be also kept in mind that those natural metabolites exhibit sometimes strong seasonal variations, and in some case are not available all year round due to the life cycle of the macroalgae. Besides, data on market value are often scarce and various prices circulating in the literature are obsolete (Values directly extracted from Tompkins, 1983) and would lead to erroneous calculations and further over or underestimation.

Polysaccharides

Alginate

This polysaccharide is sold as a salt of alginic acid, extracted from brown macroalgae. The quality of its gelling properties depends on the structure of the polysaccharide (mainly the guluronic to mannuronic acid ratio (G/M). In order to produce a high quality grade alginate for food and pharmaceutical application, you need to select a specie with a high G/M ratio. The specie chosen for biofuel production would have to reach a certain G/M ratio (such as *Laminaria*).

The average price for alginate in 2009 is 12 US\$/kg with a global market representing 318 M US\$ and 26,500 t/annum (corresponding to 95,000 t DW of brown algae).

High quality grades production still remains in Europe, with 8,900 t/annum from a few companies (FMC, Danisco, Cargill), and represent the biggest increase in terms of market share worldwide (Bixler and Porse, 2010).

It is worth noting that the theoretical conversion rate of alginate to ethanol is rather poor, around 20% (Horn, 2000) compared to other polysaccharides in brown algae (Mannitol, Laminaran, etc.). Therefore, alginate might not be the main target for bioethanol production. In turn, waste products from alginate production (floating residue) might be used for ethanol production and limit the environmental impact of alginate production (Ge, 2011).

Agar

Agar is a polymer mainly obtained from the genera *Gracilaria* spp. and *Gelidium* spp. *Gracilaria* has been successfully cultivated in Chile and Indonesia and a number of ageing, obsolete factories have closed in Europe (in Spain, Portugal, Italy, France) (Bixler and Porse, 2010). This phycocolloids is highly necessary in the pharmaceutical and biotechnology sector for its strong gelling properties. The market worldwide represent 9,600 t for an estimated value of 173 M US\$, hence the high value of 18 US\$/kg.

Setexam in Morocco constructed an 800m³ reactor in order to treat 12t of daily waste (9.85% VS) generated by agar production from *Gelidium*. The total cost of the plant was around 140,000 € (6 M BFr in 1991) and was expected to produce around 100,000 m³ per year (Morand, 1991). Another development with beneficial environmental impact is the Indonesian production of *Gracilaria* in ponds, along with milkfish, to take advantage of nutrient synergies (Bixler and Porse, 2010).

Carrageenan

Carrageenan is an umbrella term describing a family of polymers extracted red seaweeds, the complexity of chemical structures, the various purity grades, regulation and fierce competition leads to a somehow chaotic market (Bixler and Porse, 2010).

The carrageenan production reaches a total of 527 M US\$ and 50,000 t, hence a low average price of 10,5 US\$/kg. The prices range from 6 to 15 US\$/kg for the various grades of carrageenan.

Note: the recent price increase in the hydrocolloids market have been driven by high energy, chemicals and seaweed costs. The higher seaweed cost reflects seaweed shortages (Bixler and Porse, 2010) and advocates for the use of large-scale cultivation, as all easily harvestable seaweed beds reaches their limits.

Fucoidan

Fucoidan (a polysaccharide mainly composed of sulphated L-fucose) is extracted from brown macroalgae, which are of potential interest to biofuel production. Whether the processed macroalgae will be suitable for further fucoidan extraction would have to be assessed.

Prices range from 200 €/kg for some low purity material and reaches 1000 to 1100 €/kg in the pharmaceutical industry (as a coating agent for surgical application) (Hennequart F., OGT-Agence Oceanide, pers. comm.).

Proteins, peptides and free amino acids

Proteins and peptides are present in very different levels in macroalgae, ranging from 4 to 15% in European kelps (Jensen and Haug, 1956; Indergaard and Minsaas, 1991) and are subject to strong seasonal variations following nutrient supply. Algal proteins have been reported to have a low digestibility (Michel et al. 1996); this may be due to their cellular localisation or their putative associations with cell-wall polysaccharides (Kloareg and Quatrano, 1988). Protein and peptides from macroalgae

Free amino acids are widely found in numerous seaweed species, and include Alanine, Ornithine, Glutamic acid, Glycine (Holdt & Kraan, 2010).

Lipids, vitamins and pigments

Those compounds are present in relative low amount as compared to terrestrial plant. However, what macroalgae lacks in quantity, it compensates with high nutritional and economical value. Holdt and Kraan, 2010, review the Lipids (Fatty acids, Phospholipids, Glycolipids and Sterols) and pigments (Chlorophylls, Carotenoids and Phycobiliproteins) with a focus on the following genera: *Laminaria*, *Saccharina*, *Fucus*, *Undaria*, *Sargassum*, *Ulva*, *Chondrus*, *Porphyra*, *Gracilaria* and *Palmaria*, all being represented in Europe and of potential interest in biofuel production.

Phenolic compounds

Phenolic compounds such as phlorotannins, are large and complex polymers of phloroglucinol and are found in significant amount in brown algae (with the lowest content below 2% DW for subtidal species). According to Scalbert, 1991, polyphenols are potent inhibitors of methanogenesis and would therefore need to be removed prior to anaerobic digestion. Those compounds have been described as having antioxidant properties (Ragan & Glombitza, 1986) and valorisation as functional food, or food additive would be suitable.

Seaweed mill – organic improver

The complexity of extraction of metabolites, and their purification (with associated cost, regulation and environmental impact) might not suit all processor for biofuel production. A low investment solution could lie on the utilisation of the final “cake” as seaweed mill for animal feed or fertiliser. The price of seaweed mill is around 600-700 €/t DW (Hennequart F., OGT-Agence Oceanide, pers. comm.).

In its technico-economic budget, Morand (2006) presents the expenses and incomes from a methanogenic process after pressing *Ulva* and digesting the “juice”. The remaining “cake” and digestate are respectively expected to provide an income of 60 €/t of organic matter and 1€/m³ from the digestate as fertiliser.

Inhibition and toxicity (Morand, 1991)

By Product and metabolites present in the macroalgae can lead to the inhibition of the fermentation and/or anaerobic digestion as we have seen for phenolic compounds. Other substances such as heavy metals, sulphides, salts and volatile acids can inhibit the process. Though many solutions exist to stop or reduce the inhibitions.

Sulphur content in seaweed is high and ranges between 0.5 and 1% DW, and even 4.4% for *Ulva*, 4.6% for *Gracillaria* and *Fucus*. However, long retention times lead to the acclimatation of anaerobic organisms, and sulphur toxicity is a function of the presence of metals (yielding precipitates of metallic sulphides).

Heavy metals may be a problem if there are not enough soluble sulphites to precipitate them.

Sodium chloride (salt) can inhibit seaweed methanisation when suddenly introduced at 30 g/L. When the salt is introduced gradually, no perturbation is observed until up to 65 g/L. However, trials of desalting yield in a methane production decrease. Furthermore production was higher in seawater than freshwater.

Discussion on the Bio-refinery concept

The biorefinery concept from macroalgae is not new and its development is concomitant with the outbreak of World War I, and subsequent embargo on german products. At the time, Germany was the main manufacturer of chemicals, especially potash and acetone (which entered in the production of munitions). A company specialised in gunpowder production developed a process from the giant kelp that allowed them to produce both potash and acetone at the same time, alginate being an unwanted by-product. Towards the end of the war, the company was able to display a range of 54 kelp products, mainly war products, hence wasn't able to remain in business after the armistice (Neushul, 1987). However, other companies took advantage of the alginate production from the giant kelp *Macrocystis pyrifera*.

More recently, Tompkins, 1983, described a theoretical study in which with 15% of seaweed used for co-production of alginates, mannitol, and iodine, the biofuel production cost was estimated to decrease 50 to 80% for a seaweed-to-methane conversion plant.

The use of the biorefinery concept with the aim of producing biofuel (high volume/low value combined with other metabolites (polysaccharides, proteins, lipids, bio-active compounds, etc. plus derivate with low volume/high value) can be seen as either an idealistic situation or common sense of the market. Whether

the biofuel production from macroalgae will be the main product of an industry or its by-product is not clear yet. It is likely that the two situations will coexist depending on the biomass availability, the need for energy in a specific location (e.g. insular locations where energy transportation is an issue) and the evolution of the market of the various metabolites.

Biorefinery for biofuel will probably happen in time, when overlapping extraction constraint, uses of organic solvents and purification costs will be solved and compatible with a low environmental impact.

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