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PROJECT FINAL REPORT

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Name of the scientific representative of the project's co-ordinator[1], Title and Organisation:

Prof. Sammy Boussiba, Ben Gurion University of the Negev, Sede Boker, Israel

Tel: 972 8 659 6795

Fax: 972 8 659 6802

E-mail: Sammy@bgu.ac.il

Project website[2] address: www. GIAVAP.eu

[1] Usually the contact person of the coordinator as specified in Art. 8.1. of the Grant Agreement.
[2] The home page of the website should contain the generic European flag and the FP7 logo which are available in electronic format at the Europa website (logo of the European flag: http://europa.eu/abc/symbols/emblem/index_en.htm logo of the 7th FP: http://ec.europa.eu/research/fp7/index_en.cfm?pg=logos). The area of activity of the project should also be mentioned.

4.1 Final publishable summary report

Executive Summary

Microalgae produce a wide range of high-value products such as carotenoids and Polyunsaturated Fatty Acids (PUFA's) and also are a potential source of biofuel. However, significant efforts in strain development and cultivation technologies are required to reduce currently high production prices for algal biomass. Microalgae accessible to genetic engineering can also play an increasingly important role in production of pharmaceutically active substances and recombinant protein products for therapeutic or industrial applications.

The objectives of project GIAVAP were addressing the problems and challenges of genetically modulating and successfully cultivating the wide variety of microalgae by integrating universally valid biological and genetic engineering principles to redirect metabolic pathways for the efficient production of valuable algal products. The major project objective was enhancing efficiency, sustainability and profitability of future algae based industrial processes with special attention given to application of safe genetically engineered microalgae and processes for industrial exploitation. The work performed in the course of this project resulted in significant progress in important and essential tasks. Seven microalgae species were genetically engineered in the course of the project, and engineered strains overproducing PUFA, carotenoids, TAG, and high value therapeutic proteins were produced. Significant progress was achieved in understanding the physiology of high value microalgae, and advanced technologies of metabolomics and lipidomics were applied to understand the metabolic changes during transfer to starvation conditions for maximal TAG, carotenoid or PUFA production. Furthermore the challenges of large scale cultivation, harvesting and product extraction were addressed using a wide range of approaches. Algal biomass and purified bioproducts were successfully tested in various model systems, indicating a large application potential in health, agriculture and aquaculture contingent on acceptable production prices.

GIAVAP has submitted all planned 72 delivery reports (three of them only partly addressing the subject in question), 32 peer reviewed publications have been published or are in press so far, and a much bigger number of manuscripts are planned or in preparation; 105 lectures, often at prestigious international events, and many more posters and other dissemination actions have been presented. So far five patents dealing with important breakthroughs in algal genetic engineering have been granted or submitted.

In conclusion GIAVAP has produced a huge amount of novel scientific knowledge and scientific breakthroughs in genetic engineering and biotechnology of microalgae, including technical know-how to directly benefit industrial applications to the direct benefit of future algae biotechnology endeavours. Application of those findings can have dramatic positive impacts on future sustainable resource use and exploitation under creation of multiple business opportunities in neglected rural dryland areas in Europe and worldwide.

1. Project context and objectives

Microalgae produce a wide range of high-value products such as carotenoids and Polyunsaturated Fatty Acids (PUFA's) and also are a potential source of biofuel. However, significant efforts in strain development and cultivation technologies are required to reduce currently high production prices for algal biomass. Microalgae accessible to genetic engineering can also play an increasingly important role in production of pharmaceutically active substances and recombinant protein products for therapeutic or industrial applications.

The objectives of project GIAVAP were addressing the problems and challenges of genetically modulating and successfully cultivating the wide variety of microalgae by integrating universally valid biological and genetic engineering principles to redirect metabolic microalgal pathways for the efficient production of valuable algal products such as carotenoids, PUFA and recombinant proteins. A further focus was enhancing growth rate and TAG content in various microalgae towards enhancing productivities for eventual microalgal biofuels production. The major anticipated impact was enhancing efficiency, sustainability and profitability of future algae based industrial processes with special attention given to application of safe genetically engineered microalgae and processes for their future industrial exploitation. As most project results are yet to be published and confidential, the following summary will give a general overview of achievements with the attached list of publications, dissemination activities and patents giving an adequate insight into already published project achievements.

2. Scientific and Technological progress

2.1. Advances in genomics and bioinformatics

GIAVAP successfully performed genomic and transcriptome sequencing of two high value microalgae species, and analysed quantitative transcriptomics in these species under various conditions of nitrogen starvation. The findings of those studies provided not only a large number of novel and important genes for metabolic engineering of those strains, but also novel insights into the evolutionary diversity of the green-alga kingdom. Furthermore sets of miRNA were also determined and quantified under different growth conditions, implying the direct involvement of those regulatory small RNAs in regulation of gene expression for modulating metabolic processes. Supported by recent, parallel progress worldwide in genomics and transcriptomics, multiple genes of importance to TAG, PUFA, and carotenoid biosynthesis were identified, cloned and applied for genetic engineering of high value algae species.

2.2. Advances in microalgal cell and molecular biology

Project GIAVAP has demonstrated convincingly that the integrated efforts of high quality research institutions can provide dramatic progress in multiple subjects and technologies across the whole scientific spectrum of algal biotechnology to rapidly advance technical and scientific knowledge of high value microalgae.

Several species of high value microalgae were promoted to the level of well-studied model systems. Comprehensive and advanced genetic engineering technologies were developed, improved and applied for genetic engineering, creating strains with enhanced high value production or lipid accumulation. Four DNA delivery techniques and multiple novel selection markers were successfully applied to transform and genetically engineer seven different microalgae species. Furthermore the usefulness of mutagenesis for production of valuable traits was demonstrated, and algal transposable elements were identified and tested for future use in advanced genetic modification technologies.

Those molecular technologies were supported and complemented by application of the most advanced technologies in metabolomics and lipidomics for in detail understanding of metabolic and physiological changes in microalgae. Among others critical bottlenecks in metabolite flows and enzymatic activities during TAG biosynthesis induction have been identified in this way. Furthermore proteomics technologies were applied for the isolation of proteins of importance in TAG biogenesis.

2. 3. Advances in microalgal cultivation and biotechnology

In parallel, project GIAVAP also provided further insight into algal growth physiology for lipid or high value product accumulation, by lab scale and pilot scale outdoors cultivation for strain evaluation in different species, and first time pilot scale outdoors cultivation of genetically modified microalgae strains. Several strains were cultivated in tubular, panel and raceway reactors at the 100 to 5000 liter scale. Biomass was harvested, products were extracted by biorefinery approaches and tested in a number of models for their usefulness in health care and aquaculture. Important findings were achieved in high value product extraction from microalgal biomass. Significant findings confirmed the usefulness of algal products in medical, aquaculture and agricultural applications.

2. 4. Specific advances in the biotechnology of high value microalgal species:

a) *P. tricornutum* genetic engineering and biotechnology.

The most complete set of experiments was dedicated to developing the biotechnology of the diatom *Phaeodactylum tricornutum*. This was the only high value microalgae accessible to genetic engineering at the onset of GIAVAP that also accumulates high value products (LC-PUFA and fucoxanthin) and TAG. Nevertheless, physiology and biotechnological potential of this species had been studied to a lesser extend compared to other algae species. GIAVAP has advanced this species to a completely understood model species, by adding in detail physiological, lipidomics and metabolomics studies to the large array of existing molecular knowledge. Genetic engineering was applied to overexpressing a number of genes for enhancing substrate flows towards desired products. These experiments resulted, among others, in several-fold overexpression of the high value keto-carotenoid fucoxanthin; significantly enhanced DHA productivity, and increased TAG productivity and content. Various highly expressed and inducible endogenous promoters were isolated, tested and applied with promising results.

Functional human proteins such as EPO and growth hormone were successfully expressed in *P. tricornutum*, and tested in biological assay systems, confirming the suitability of this species to act as green factory for recombinant protein expression.

Detailed metabolomics and lipidomics studies allowed assessing the metabolic adjustments in *P. tricornutum* during conditions such as nitrogen starvation. The same technologies have also been applied to understand the impacts of various genetic engineering approaches on the metabolic fluxes inside the cell. Finally, *P. tricornutum* was successfully cultivated in pilot size outdoors reactors, and TAG, fucoxanthin and EPA productivities were determined. Methods for extraction and purification of fucoxanthin were tested. Different isolates were compared to determine the most suitable strains for outdoors cultivation and biomass production. Genetically engineered strains produced by GIAVAP partners were also cultivated outdoors, after seeking and receiving the necessary permits from the relevant authorities.

b) Development of *Parietochloris incisa* as high value model species

The chlorophyte *Parietochloris incisa* (recently reclassified as a member of the genus *Lobosphera*) is the only microalga reported to produce ARA in large quantities, accumulating up to about 20 % of dry weight and 60 % of TFA under nitrogen starvation conditions, making this alga the richest plant source for arachidonic acid. More than 90% of total ARA is deposited in the TAG storage lipids. GIAVAP has made significant advances in genetic engineering, molecular characterization, physiology and metabolic analysis of this microalga to the extent that this species can now be considered a well understood model organism.

GIAVAP has determined a high quality genome, together with transcriptome sequencing and quantitation under four different growth conditions. In addition to a wide range of information relating to stress and lipid metabolism, the genome also revealed all the genes required for sexual reproduction and motility, phenomena confirmed by recent observation of motile zoo-spores under certain growth conditions. Functional annotation of the genome is being curated by a team of experts through a web interface. In parallel, a set of microRNA were determined and quantitated, again under several growth conditions, and quantitive microRNA and transcriptome sets wait to be compared for assessing possible regulatory miRNAs affecting TAG, PUFA and carotenoid biosynthesis.

The strain has been successfully transformed using three different selectable markers, and genetic engineering was demonstrated by rescuing the P127 mutant (deficient in the delta-5 desaturase), by transformation with the delta-5 desaturase wt gene to restore arachidonic acid biosynthesis. Endogenous dominant selection markers for this gene were developed that will allow engineering the strain by self-cloning approaches.

In parallel, the strain has been subject to a wide range of metabolomics and lipidomics analyses under normal and nitrogen stressed growth conditions, to reveal the full extent of metabolic changes occurring during stress induction leading to TAG biosynthesis. This set of lipidomics and metabolomics data will now serve to develop detailed models of metabolic readjustments and lipid biogenetic pathways in this species under various conditions. Production of the high value fatty acid DGLA by the *P. incisa* mutant P127 was demonstrated successfully using tubular or panel photobioreactor systems.

c) Advances in *H. pluvialis* cell and molecular biology

H. pluvialis is one of the few commercially cultivated microalgae species, due to its production of the high value carotenoid astaxanthin, and BGU has developed the biotechnology for its commercial exploitation now applied by AlgaTech in Qetura.

GIAVAP has advanced this species towards its full biotechnological potential by genome and transcriptome sequencing, developing advanced methodologies for genetic engineering of the nuclear and chloroplast genomes, and by advancing our knowledge on the metabolism of stress induction and oil globule formation and translocation. Strains with significant astaxanthin overproduction were created by genetic engineering. Genetic engineering approaches based on self-cloning, by inserting of rearranged *H. pluvialis* DNA only, have been developed, resulting in strains with significantly improved properties for biotechnological exploitation that may be applied widely and with little restrictions on their cultivation and product use. The major breakthrough here is that genetic modifications were performed using a novel advanced transformation vector allowing inserting two transgenes simultaneously using an endogenous dominant selection marker. Using linear molecules for transformation, we demonstrated the ability to create self-cloned improved strains without any foreign DNA inserted.

The interaction between starch and TAG biosynthesis after application of nutrient stress was studied and analysed and modelled by the help of metabolomics and enzyme activities during several time points of nitrogen starvation. Those data were integrated into a model for system analysis of the physiological processes in this alga that may afterwards be joined with transcriptomics and other results for a full systems biology analysis.

d) Advances in *Nannochloropsis oceanica* genetic engineering and biotechnology

1) Transformation of *N. oceanica*: Transformation of *Nannochloropsis sp*. has recently been reported by Kilian et al. (2011) using selectable markers for blasticidin, hygromycin and zeocin resistance. The recently published *Nannochloropsis oceanica* genomic sequence has been used to identify the necessary promoter and gene sequences to construct plasmids for transformation of this economically important oil producing algae. The materials and methods described in the publications and by the group of Prof. Christoph Benning (GIAVAP advisor) were used to transform this commercially important species. Transformation efficiencies achieved were very high allowing production of large sets of modified clones for biochemical analysis.

2) Modulation of lipid metabolism by genetic engineering: This transformation technology has been successfully used to engineer the lipid metabolism of *N. oceanica.* A putative desaturase gene mediating conversion of oleic acid (18:1) to linoleic acid (18:2) in extraplastidial lipids was cloned from *N. oceanica* and overexpressed in the same strain, driven by the lipid droplet surface protein (LDSP) promoter sequence. The cloned gene seems to be functional in heterologous and homologous transformation systems and its overexpression in *N. oceanica* lead to substantial alterations in the ratio of 18:1 to 18:2. Furthermore, some increases in the relative percentage of major LC-PUFA were observed in the transformant lines relative to the empty vector control (EV).

3) Physiology of growth and lipid accumulation in *Nannochloropsis* sp: Growth and physiology of lipid accumulation in *Nannochloropsis* were studied intensively in two laboratories, and compared to that of various *P. tricornutum* strains. The most productive lipid and biomass producers among all strains investigated were *Nannochloropsis* belonging to two distinct species as classified by phylogenetic analysis. Significant differences in biomass and lipid productivities were observed within different isolates of the same species. In detail analysis of one-stage and two stage cultivation indicated a wide margin for productivity optimization achievable dependent of strictly defined growth and starvation conditions and protocols. Physiological plasticity of *N. oceanica* in response to

varied salinity levels has been investigated, and its impact on cellular metabolism has been determined.

d) Mutagenesis for stress and herbicide resistance

Chemical mutagenesis was applied successfully to create *Nannochloropsis* strains for improved performance in large scale cultivation. Herbicide and oxidative stress resistant strains were created and tested successfully, indicating that classical mutagenesis approaches in many instances can provide faster and simpler solutions to improve strain performance than genetic engineering.

e) Genetic engineering in other species

In addition to the above species, genetic engineering tools were developed and tested by GIAVAP partners for other species as well. Advanced transformation tools for enhanced protein expression in *C. reinhardtii* were developed. Transformation of *Chlorella sp* by *Agrobacterium* mediated gene transfer was developed, and *Ostreococcus taurii* was also successfully transformed. This brings the total of algal species successfully transformed to seven, from the clades Chlorophycea, Trebouxiophycea, Diatoms and Eustigmatophytes.

Those results indicate that careful testing and development of transformation technologies based on using a variety of promoters, selectable markers, gene delivery technologies and especially suitable cultivation and recovery technologies can result in successful transformation of essentially all microalgae species. Thus it can be expected that shortly a large number of genetic engineering approaches will be available to transform the wide range of potential high value algae species.

2. 5. Integration of project achievements for advancing microalgal biotechnology

In collaboration with experienced labs in metabolomics and lipidomics, GIAVAP progress has permitted transforming any microalgae to an adequate model for developing high value or biofuels production as demonstrated in the examples of *P. tricornutum*, *P. incisa*, *H. pluvialis* or *Nannochloropsis*. GIAVAP has demonstrated, by means of genomics and transcriptomics analysis, focused efforts to developing transformation and genetic engineering technologies, and application of experienced teams for studying cell physiology, metabolism and lipid biosynthesis pathways, that the necessary knowledge to advance any species to a fully understand industrially applicable microorganism can be created within a few years and modest resources.

3. Dissemination and Exploitation of Project Results

GIAVAP has submitted all planned 72 delivery reports (with three of them only partially achieving or addressing the subject in question). As outlined in the list below, 32 peer reviewed publications have been published or are in press, and a much bigger number is planned or in preparation. 105 lectures have been given by scientists of GIAVAP participants, many of them in prestigious international meetings, often as invited plenary speakers. Furthermore numerous poster presentations and other dissemination actions have been presented, many of them by junior research staff and graduate students participating in important international and national scientific events, significantly contributing to their training and education. Five important patents so far have been granted or submitted in the emerging field of microalgal high value and biofuels technology, some of them presenting blueprints for key methodologies in creation of genetically engineered microalgae useful for high value products and biofuels. A huge amount of confidential know how has been developed and shared between project partners, making GIAVAP the centre of a renewed push to excellence in European microalgae biotechnology, widening the scope from a previously narrow focus in outstanding *C. reinhardtii* oriented cell and molecular biology to a much wider spread effort on genetic engineering and cell biology in several high value algae species from different kingdoms. It is expected that those achievements will be transformed into industrial applications in several fields such as fuel, PUFA, carotenoid production and others. A detailed list of publications and dissemination actions is attached below.

In conclusion GIAVAP produced not only a huge amount of novel scientific knowledge with profound scientific breakthroughs in genetic engineering of microalgae, but also significant amounts of scientific and technical know-how to directly benefit industrial applications in multiple fields to the direct benefit of future algae biotechnology endeavours. Application of those findings can have dramatic positive impacts on future sustainable resource use and exploitation under creation of multiple business opportunities in neglected rural dryland areas in Europe and worldwide.

4. Economic and Societal Potential of GIAVAP Progress

Microalgae are being considered a potential high impact feedstock for addressing future needs for fuel, feed, food, high value additives, bulk chemicals and materials, and they also provide adequate options for water treatment and bioremediation, with a combined market potential in the trillion dollar range. However, none of those potentials is being exploited at any significant scale, and the few algae-based enterprises combined (excluding R&D and startups without market oriented revenue) have combined sales of about 1.25 billion dollars yearly, exclusively in high priced niche markets. The potential of microalgae for competitive production of medium to high value products such as PUFA and carotenoids is significant. As demonstrated by GIAVAP market analysis and assessments, a number of potential products representing billions in market volume are within the reach of production prices achievable by microalgae, subject to sustained growth yields in large scale facilities and adequate extraction and purification approaches, if production price can be reduced 2 – 5 fold by the help of enhancing product content and achieving stable, moderately enhanced growth rates.

Current state of the art

The microalgal biotechnology laboratory (Sede Boker, Israel) currently applies variations of the so-called hybrid cultivation approach, preparing high quality inoculum in panel reactors containing disposable plastic lining under controlled conditions, while preparing the bulk amount of biomass in race way ponds in batch mode. This allows cultivating either under nutrient replete or under starvation conditions for varying biomass compositions, and it also avoids many contamination and management problems arising from (attempted) continuous cultivation approaches. Recent MBL experiments indicate that this hybrid approach can yield 20-25 g m^{-2} day^{-1} of oil rich *Nannochloropsis oceanica* biomass except for the two coldest winter months. Our experience with other strains indicates that this approach is viable for efficient cultivation of most species under investigation, and allows adaptation of growth conditions for achieving the desired product contents and biomass qualities.

Production costs for algal biomass are difficult to estimate due to the wide variety of technologies applied, different species specific cultivation requirements, the lack of large production facilities and the lack of published information from commercial producers. Currently available cost estimates are often over-optimistic extrapolations from pilot experiments and must be considered with care. Some essentials concerning high value production prices can be deduced by exploiting knowledge from commercial microalgae production, e. g. *H. pluvialis* at the Qetura facility or *Spirulina* production in open raceways by Earthrise. Based on economic output at Qetura, a production price of at least 75 Euro per kg dry weight of *H. pluvialis* in two stage cultivation may be deduced in this small commercial facility (Boussiba, personal communication). Considering one stage cultivation of a robust and fast growing strain in a significantly larger facility, a price of less than 20 Euro per kg may be extrapolated for tubular reactor facilities. A recent assessment by project Aquafuels (www.aquafuels.eu), incorporating recently published economic and environmental assessments, calculated a 10 Euro production price per kg dry biomass in tubular PBRs in cases where CO_2, water and fertilizer have to be bought, less if such resources are available on site. Similar costs have been calculated for panel reactor production using UNIFI's technology and product yields, assuming availability of adequate cooling water supplies.

Production costs for raceway production are about Euro 1.8 per kg algal biomass if all resources (water, CO_2, fertilizer) have to be bought at full value. If on site resources are available at lower cost those costs could be significantly reduced. However, it will have to be demonstrated whether use of waste water and waste nutrient sources can yield algal biomass for anything but biofuels, due to potential contamination and health risks involved.

Processing and biorefinery:

Processing and purification costs of microalgal products are as yet poorly assessed at scale and will vary from strain to strain. The minimal processing required for production of a marketable product from microalgae is harvesting, grinding, and drying of the biomass for production of algae meal applicable in animal and fish feed, specifically as astaxanthin, lutein, or PUFA supplement. Since PUFA, lutein or astaxanthin contents in algae powder are far above the animal's feed requirements, supplementing low priced soy and corn based bulk feed with crude algae powder would suffice for strengthening feed with essential pigments, vitamins, fatty acids and microelements, with a significant increase in total feed value. This is probably the easiest accessible bulk market for large scale application of algal biomass that does not require expensive biorefinery and purification steps. Where purified products promise better returns, existing separation and purification technologies for carotenoids, fatty acids and other components can be adapted, whereby the rather high product concentrations found in microalgae (compared to other sources) facilitate the required purification procedures. Processes for refining of wet biomass may have significant advantages, as significant drying and energy costs can be saved.

Sustainability of microalgal high value production:

The environmental assessment of algae-based products can be based on recent LCA analyses for tubular reactor and raceway pond production presented by several authors supported by a large comparative assessment of published LCA analyses done by the Aquafuels project (http://www.aquafuels.eu/deliverables.html). Raceway-based algal biomass production can be environmentally beneficial or neutral in terms of energy and GHG balance, if all the algal biomass is being exploited as feed or for energy production. Chemicals produced by GHG and energy neutral raceway technologies, such as EPA from *Nannochloropsis*, DHA from *Isochrysis*, lutein from *Scenedesmus* will therefore easily out-compete competing products from agriculture or extracted from the oceans by unsustainable fishing practices. Such arguments can serve as important marketing arguments, taking full advantage of the green label ‘algae’.

In contrast, tubular PBR production will consume significantly more resources than can be produced in the form of biomass and cannot be considered to provide environmental benefits.

Therefore, products raised in expensive photobioreactors, possibly under artificial illumination or under heterotrophic conditions cannot make such claims without the complex task of actually performing a comparative all inclusive LCA.

Impact of genetic engineering on marketing prospects:

Genetic modification can further favor the microalgal products by reducing expected production prices by 50% or more, as evidenced by achievements to date such as increases in carotenoid and PUFA contents by 50 – 200%, which can be further enhanced by additional modifications for more efficient cultivation and production. Theoretically, productivities of PUFA, astaxanthin, fucoxanthin or lutein, can be doubled or tripled by using 1 – 3 subsequent genetic modification steps of those achieved by GIAVAP partners. Nevertheless, the economics (and sustainability) of production in closed PBRs required for the cultivation of such genetically modified microorganisms, will remain poor. However, the application of safe GMO-technologies such as mutagenesis or self-cloning, facilitating the licensing of outdoors cultivation of the resulting GE strains may result in product prices and environmental impacts below those of any competing products. BGU has submitted a patent on self-cloning strategies in *H. pluvialis* using dominant endogenous markers and rearranged algal DNA only for increasing astaxanthin productivity by genetic transformation. It therefore appears possible both to significantly improve product yields and to achieve sustainability of microalgal high value products and bulk biomass in the near future, using safe genetic modification technologies for outdoors raceway cultivation.

5. Contribution of GIAVAP achievements to development of biofuels production by microalgae

1) Enhancing sustainability and yields by enhancing growth and oil content

Microalgae may address future needs for fuel, feed, food, high value additives, bulk chemicals and materials, with a combined market potential in the trillion dollar range. The huge efforts invested recently in investigating this potential have significantly advanced our understanding in algal biology, cultivation, harvesting and biomass processing, but no commercially viable sustainable production pathways have yet resulted from those efforts. Current state of the art in microalgal mass cultivation suggests annual biomass productivities of around 50-60 dry tons per hectare resulting in about 15 tons of biofuels. As modelled by Stephenson et al (Fig. 1) such biomass and fuel yields would recover all energy invested, and result in little GHG emissions savings compared to fossil fuels, but would not qualify as renewable fuels under the upcoming standards of the EU and US.

Energy Fuels **2010**, *24*, 4062–4077 · DOI:10.1021/ef1003123 *Stephenson et al.*

Table 4. Fossil-Energy Requirement and GWP of Biodiesel from *C. vulgaris* Cultivated in Raceways, Considering Modifications to the Base Case[a]

	fossil-energy requirement		GWP	
scenario	GJ/ton of biodiesel	savings (%)[b]	kg of CO_2 equiv/ton of biodiesel	savings (%)[b]
		Base Case		
raceway base	6.5	85	713	78
		Modifications to the Base Case		
productivity by area				
(base = 40 tons ha^{-1} $year^{-1}$)				
80 tons ha^{-1} $year^{-1}$	−0.1	100	331	90
20 tons ha^{-1} $year^{-1}$	19.9	54	1482	54
10 tons ha^{-1} $year^{-1}$	46.7	−8	3023	6

Fig. 1: sustainability criteria (GHG emissions and fossil energy savings) of microalgae cultivation for biofuels production under current productivities.

A variety if GIAVAP findings as described above, both in understanding of algal physiology, up-scaling and genetic engineering can significantly improve the environmental and economic balance of algal biomass production. Recent MBL experiments indicate annual biomass productivity of 60 – 70 tons per ha and year and lipid productivity of near 20 tons per ha and year. We identify significant optimization potential at the level of culture management and optimization of panel productivities by means of engineering, with the ultimate productivities using non-modified algae remaining to be determined. Such yields are adequate for production of feed or high value products. However, the renewable fuels standards of the EC and US require significantly improved return on invested energy, and strongly reduced GHG emissions compared to fossil fuels. Key difficulties to be overcome are in reducing the production and processing costs of algal biomass, by means of progress in engineering and culture management, and by selecting and genetically improving strains for maximal productivity at minimal costs.

GIAVAP has managed to induce significant gains in productivities of various high value metabolites and TAG accumulation. In addition, technologies propagated to enhance overall biomass productivities are also being tested. Currently the GMO technologies that may most significantly affect biofuels productivity are:

- Proprietary technologies for self-cloning of microalgae for facilitated licensing and cultivation outdoors;

- Enhanced TAG accumulation with so far three approaches successfully enhancing TAG content of algal biomass;
- Enhancing TAG productivity by applying inducible promoters.
- Enhancing growth or biomass accumulation by means of reducing antenna size, or by improved inorganic carbon assimilation according to published technologies is underway;

Those genetic improvements as described above could have the potential to increase biomass and fuel production to 150 tons per hectare and year, or 60 tons per ha and year respectively, with two to three subsequent modification steps. Only such success might allow production of algal biomass and biofuels at a cost below $1.- per kg, with ROI and GHG savings beyond the requirements of various renewable fuels standards (Fig. 2).

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		Base Case		
raceway base	6.5	85	713	78
		Modifications to the Base Case		
productivity by area (base = 40 tons ha^{-1} $year^{-1}$)				
80 tons ha^{-1} $year^{-1}$	−0.1	100	331	90
20 tons ha^{-1} $year^{-1}$	19.9	54	1482	54
10 tons ha^{-1} $year^{-1}$	46.7	−8	3023	6

Fig. 2: Production of around 80 tons of biofuels per hectare and year is expected to provide almost GHG neutral biofuel with almost 90% savings on invested fossil energy.

<u>2) Enhancing the economics of biofuels production by increasing by-product content</u>

Costs for raceway production have been estimated by project AquaFuels at about Euro 1.8 per kg algal biomass if all resources (water, CO_2, fertilizer) have to be bought at full value (see above). BGU considers this an optimistic estimate achievable in the more distant future only. If on site resources are available at lower cost production costs could be significantly reduced.

Recent achievements by GIAVAP may however provide approaches for improving the economics of algal biomass production thanks to enhancing high value product contents. Carotenoid content was significantly increased in *P. tricornutum* and *H. pluvialis*, by single gene overexpression. This technology seems to be universally applicable and can give rise to strains with over 1% - 2% carotenoid content. A combination of a few genes overexpressed appears to suffice to significantly enhance EPA content in *Nannochloropsis* biomass. If *Nannochloropsis* with 3% EPA (or *Chlorella* or *Scenedesmus* with 0.5% carotenoid) have a gross value of about $ 5.- per kg dry biomass, doubling the EPA or carotenoid contents in those species would increase biomass value to around $ 10.- per kg, which would be significantly above estimated production costs of currently around $ 5 per kg biomass. Covering projected global demand for high value carotenoids and LC-PUFA using such algae would cover up to 10 % of global fuel demand in the form of algal waste biomass available as free feedstock for biofuels production. Biorefinery approaches for extraction and separate marketing of high value products and valuable by-products will however be essential for improving the economic balance of the production processes. Such new approaches need to be developed and tested for various product categories and strains to significantly reduce products extraction costs.

3) The importance of safe GMO technologies for large scale algae production

In order to profit from possible benefits of GMA microalgae, outdoors cultivation in open ponds must be applied. Microalgae are not included in most current regulatory EU documents concerning genetic engineering. A recent enquiry with the EFSA confirmed that for most matters of biological safety microalgae can be considered microorganisms, such that genetically modified microalgae would fall under regulations in the "Guidance Document of the EFSA GMO Panel on the risk assessment of genetically modified microorganisms and their derived products intended for food and feed use" (http://www.efsa.europa.eu/en/efsajournal/pub/374.htm). Nontoxic non-pathogenic GMO algae fall under biosafety class 2 (apparently class 1 in Germany) and require cultivation in contained enclosures. Outdoors cultivation without a lengthy licensing and testing period is not permitted. Accordingly, so far no GMO algae are cultivated outdoors anywhere.

However, genetically modified microalgae can be used without restrictions if they are the product of mutagenesis, cell fusion (including protoplast fusion) including production of hybridomas and plant cell fusions, or self-cloning consisting in the removal of nucleic acid sequences from a cell of an organism followed by reinsertion of all or part of that nucleic acid. This could include reinsertion of mutated algal genes used as dominant selectable markers as demonstrated by GIAVAP.

6. Expected Impact

6. 1. European competitiveness in algal biotechnology

Project GIAVAP has restored some of the lost competitiveness in algal biotechnology to European R&D. Unfortunately, most of GIAVAPs fruit will be harvested in the US and China, entities that maintain and promote far more competitive algal R&D programs and will willingly absorb the results reported by GIAVAP to promote their own economic development. China, essentially inexistent as a serious research contender when GIAVAP was conceived, is now publishing high quality algae research papers on a regular basis and investing massively into algal biotech enterprises for the nutraceuticals and high value production, and in aquaculture.

In Europe, to the contrary, the new Horizon 2020 program, published after a full years break of no funding at all, seems not even to mention the word algae. Similarly, GIAVAP partners have not received any feedback from the large European industries concerning interest in microalgae, and microalgal industry efforts are left to the few SMEs dealing with a number of minor niche products, slowly being outcompeted by overseas competition. While in the US, both government and large industries have been pumping hundreds of millions of dollars yearly into microalgal R&D with the hope of creating a local biofuels and biomaterials industry, with significant successes, the same investments in Europe lie in the 10% range. It is most likely that a significant number of trained GIAVAP professionals will find their way to the US or elsewhere abroad in the face of lacking European dynamics. It must be clearly stated that participating GIAVAP SMEs, instead of creating a bridge towards large scale investments into novel business opportunities, chose to remain in their hedgehog position of defending their own turf, while others chose to sell of their assets to overseas interests.

6. 2. Sustainable production of raw materials

Above shortcomings are specifically disappointing since GIAVAP has clearly demonstrated that microalgae can provide sustainable supplies of high value products contingent of the necessary investments, funds and government support. The approaches suggested above can provide high value materials such as PUFA or carotenoids at competitive prices based on novel industries in southern, marginalized dryland areas of Europe, supplemented by feed or fuel products to create GHG neutral productive capacities that can replace unsustainable production in fishery and agriculture and reduce imports to lower pressure on land and biodiversity in tropical areas. The projected environmental services derived from sustainable local production would justify adequate subsidies and compensations as well as a ‘feed in tariff’ like promotion to allow the European algal industry to position itself competitively in the global market.

6. 3. Business opportunities in remote and neglected areas:

Alga biotech industry would be best established in the Southern parts of Europe and the associated Mediterranean countries, with ample sunshine and large areas of underused dryland. Considering the fact that those areas suffer of marginalization, lack of investments and business opportunities, novel algal biotech companies based on GIAVAP progress, similar to GIAVAP participants AlgaTech and AlgaFuels, could draw quality investments and workforce into economic problem areas and as such promote and restore economic growth. This is clearly beyond the capabilities of a mostly research oriented FP7 consortium, but a task for policy makers at the European and Mediterranean level, considering the benefits of sustainable biomaterials production nearby contrasting to import of unsustainable supplies from remote areas of the world.

GIAVAP PARTNERS and LINKS:

Nr	Participant Organization Name	Participant short name	Country
1	Ben Gurion University of the Negev (BGU)	BGU	Israel
2	Rothamsted Research (RRES)	RRES	UK
3	J.W. Goethe Universität Frankfurt (GUF), Germany	GUF	Germany
4	Georg-August-University, Göttingen (UGOE)	UGOE	Germany
5	University College, London (UCL)	UCL	UK
6	A4F-AlgaFuel, S.A. (AlgaFuel)	AlgaFuel	Portugal
7	Rosetta Green (RGen)	RGen	Israel
8	Le Mans University, France (UM)	UM	France
9	CNRS/University Pierre et Marie Curie (CNRS)	CNRS	France
10	University of Firenze (UNIFI)	UNIFI	Italy
11	Algatech (ALGATECH)	ALGATECH	Israel
12	Nimrod Shaham & Amos Zamir C.P.A.	SHAHAM CPA	Israel