PROJECT FINAL REPORT

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Executive Summary

The D-Factory CO_2 Microalgal Biorefinery project has made enormous strides in reducing the technical bottlenecks to produce high added-value products for food, feed, cosmetics and nutraceutical markets, strengthen the competitiveness of the European marine biotechnology industry and increase the likelihood for investment in a CO_2 microalgal biorefinery.

Halotolerant, hyper-accumulating carotenogenic strains of *Dunaliella salina* have been cryopreserved, patented and characterised and strains DF15 and DF40 have been deposited with an International Depository Authority. Results from tests of carotenoids extracts and defatted algal powders suggests demand for D-Factory products may be substantial. This could be met, sustainably, with current D-Factory know-how. Data from operating a state-of-the-art raceway in Eilat has been bench-marked and the accumulated cultivation know-how used to facilitate development of a demonstration pilot cultivation plant owned by Monzon Biotech. The know-how at Monzon now includes capturing flue gas CO_2 from the combustion of natural gas for heat and power; controlling seasonal temperature variations for year-round cultivation in more northern hemispheres with heat/cooling systems and integrating production with a salt mining facility. It also includes how to double algal biomass yield and control predators; how to integrate *Dunaliella* cultivation in PBRs for inoculation purposes; how to culture in brine with the correct composition and how to stimulate production of the colourless carotenoids phytoene and phytofluene using new generation herbicides. A novel cultivation method for producing *Dunaliella* algal powders with a more than 2-fold increase in one of the more unusual carotenes, *9-cis* β-carotene, targeted as the active compound in treatments for diseases related to retinoids, now paves the way for using D-Factory technologies to produce extracts enriched in 9-cis β-carotene for future clinical trials.

Current practice for harvesting *D. salina* from saline culture medium uses well-established disc-stack centrifuges, but the cell wall-less cells are easily ruptured, which increases the organic matter content of harvesting effluent and increases costs for effluent treatment before discharge to water-courses. To drive costs down, D-Factory partners have developed the Evodos T50 spiral plate centrifuge for continuous harvesting (>90% cells intact), and tested pre-concentration with membranes to reduce energy costs, costs in water treatment and waste.

Carotenoid extracts have been obtained after processing powders with supercritical CO_2 and formulated in a variety of ways for tests by stakeholders in different beverage-type applications. A method using solvents to separate 9-cis β-carotene from other carotenoid isomers was developed; carotenoid co-products are well-suited as natural colorants for markets that are growing to meet demand from the LOHAS demographic and other food sectors. The defatted powder shows great promise as a feed additive and also as a protein-rich bulking agent in fish sausages, and the individual starch, polar lipids and protein fractions have been further processed as food additives, emulsifiers, and in gluten-free protein-enriched bread as prototypes for stakeholder development.

Data from technologies developed in the D-Factory project have been analysed using an exceptionally comprehensive range of methodologies covering all relevant environmental, economic, social, technological and regulatory aspects. The insights gained have been condensed into lessons learned with concrete recommendations on how to improve the sustainability of algae cultivation and use in general and nutraceutical production in particular. The D-Factory value chain has been described and modelled and the business case developed for investors. The D-Factory Platform Tool offers stakeholders training of the concepts behind the D-Factory algae biorefinery and is available on the D-Factory website, which also hosts newsletters, press releases, oral presentations, reports, videos and outcomes of the project. Outputs have been widely disseminated via many European and international conference presentations to encourage future developments for sustainable production in the algae industry in general.

Project Context and Objectives

The D-Factory project "*The CO_2 micro algae biorefinery*" sought to demonstrate the requirements for establishing a sustainable, CO_2 algae biorefinery as a world benchmark, based on the cultivation and processing of the alga *Dunaliella salina*. The alga *Dunaliella salina* was chosen because it grows well in highly saline non-potable water across the globe, using CO_2 and solar energy. It is also the richest source of natural orange, yellow or red pigmented carotenoids known and represented an ideal starting point for drawing in European innovations in biomass processing technologies to produce extracts of natural colorants as well as a range of natural carotenoid isomers for treating atherosclerosis, diabetes, psoriasis and ophthalmologic diseases.

By fractionating the algal biomass into carotenoid isomers, glycerol, and carbohydrate-rich defatted powder, the project aimed to produce multiple bio-based products, each able to replace fossil-based products. By understanding how to tailor production to deliver competitive end products to the potentially multiple end user markets, there should be minimal waste and, by integrating the upstream and downstream technologies in a robust, reliable and sustainable supply chain it should be possible to deliver a demonstration pilot that would stimulate commercial investment and serve as a model for replication globally.

The objectives of the project that were established to deliver the D-Factory biorefinery and the context within which they were set before the beginning of the project were as follows:

1. ***Establish a D-Factory reference library of at least 12 new monoclonal halotolerant microalgal strains isolated either from partner/ stakeholder locations or developed by mutagenesis, cryopreserve where possible and make this available for D-Factory Partners to screen, characterise and use in the D-Factory demonstration of a microalgal biorefinery.***

Culture collections have typically sourced *Dunaliella* for β-carotene and lipid production, but many of these strains have been in culture for >10 years, some type strains for over 50 years, and none has been screened or characterised for their production potential and use in a microalgal biorefinery. Moreover, there are strains of *Asteromonas* and potentially other halotolerant genera that grow under the same conditions as *Dunaliella* and are very similar in physiology, which could provide the basis for a profitable biorefinery either separately or in combination with *Dunaliella*.

2. ***Establish production protocols that enable suppliers of microalgal biomass to tailor their production of halophytic algae such as Dunaliella using techniques based on strain selection and/or cultivation, in order to meet biorefinery specifications.***

The molecular constituents and biochemical pathways expressed by *Dunaliella* have largely not been investigated in a systematic fashion. No *Dunaliella* genome has been completely sequenced, and the majority of the molecular mechanisms employed by the alga to regulate its component metabolic processes are unknown.

3. ***Benchmark an open raceway facility producing kilogram quantities of Dunaliella biomass in terms of its technological, economic, environmental and social sustainability.***

NBT is producing *Dunaliella* on a commercial scale in large outdoor open raceways where natural environmental conditions satisfy algal growth conditions and sophisticated biotechnology is used to control all factors affecting its cell growth and chemistry. Its technology is aimed at producing a β-carotene-enriched source of biomass. Capital costs associated with cultivating *Dunaliella* in open ponds, harvesting the biomass using Westfalia centrifuges, and dewatering and spray-drying the biomass for sale as a β-carotene-enriched product that meets FDA requirements are known by

NBT, along with costs for engineering, permitting, infrastructure preparation, plant installation and integration, and contractor fees. Operations and maintenance costs for cultivating *Dunaliella* (expenses for nutrients (N-P-K), CO_2 distribution, and water replenishment due to evaporative losses, as well as utilities, components replacement, and labour costs) are also known. However an integrated assessment of sustainability has not been undertaken before.

4. ***Develop and implement a pilot (10 m3) cultivation PBR which, by using controlled conditions, is capable of producing large quantities of high density (2.0 g/l DW) microalgal biomass tailored to meet D-Factory biorefinery demands, offers sustainable scalability to industrial scale (several hectares), provides data for assessment and modelling, and Informs the creation of the D-Factory business plan***

A4F has built the largest PBR in the world but this has not been designed for cultivating halophytic algae to meet biorefinery requirements. Several types of PBR have been designed and tested since the late 1940s. All parameters (e.g. nutrients, light regime, gas exchange) can be technically designed to achieve optimal culture conditions and microbial containment, and to minimize the risk of contamination. However, since the installation and operating costs of PBRs are orders of magnitude more expensive compared to open raceways, their use tends to be restricted to producing biomass as a source of aquaculture feeds, food additives, speciality chemicals, and cosmetics and as research tools for biofuel production. Hybrid cultivation using a combination of PBRs and open ponds has been proposed for large scale algae production, but not implemented.

5. ***Develop and demonstrate cell harvesting technologies to process ~2,500l saline water /h and recover at least 90% intact Dunaliella cells at lowest cost from open ponds or PBRs for the D-Factory demonstration***

Dunaliella does not have a rigid cell wall so cytoplasmic cell contents can easily be lost in the course of harvesting from large volumes of water. The Evodos harvesting technology has been tested on halophytic *Dunaliella* strain(s) and a recovery of >90% live cells, 5.6% dw basis carotene, 29% dry matter was achieved but at small throughputs < 250 l/hr.

Harvesting of microalgae by membrane micro/ultrafiltration is a proven technology but has not been applied to the more fragile cells of *Dunaliella*. The literature reports difficulties in harvesting microalgae using membrane technologies, *Dunaliella* in particular, because the process is typically operated under controlled pressure difference conditions, with harsh fluid stress conditions, which compromise cell integrity.

6. ***Develop methods for microalgal cell storage, handling and disruption and for microalgal bioprocessing using technology schemes based on scCO2, HPCCC, and membrane technologies, to produce extracts/fractions for bio/chemical profiling, and bioactivity screening, and for D-Factory scale-up designs to industrial scale, taking into account technical, environmental and economic aspects using data obtained from mobile units installed at the D-Factory demonstration production site.***

Methodologies from the 1980's based on fractional vacuum distillation with ethanol, non-polar solvent extraction and chromatography are well-documented to extract glycerol, carotenes and algae meal from *Dunaliella*. However moderate to low titre components with good bioactivity can be missed. Also, large scale preparation of target compounds (carotenoids, PUFAs) is currently done using liquid-solid chromatography (LSC) but this is an expensive procedure consuming large quantities of solvent. A range of innovative industrial-scale technologies is now available that could be extremely efficacious in processing *Dunaliella* for the range of compounds that it possesses:

- High Pressure Countercurrent Chromatography (HPCCC), which is uniquely suited for the large-scale purification of lipophilic, unstable target components such as carotenoids and

PUFAs. The quantity of organic solvents required in HPCCC can be as much as 10-fold less than Liquid Solid Chromatography;

- Supercritical CO_2 ($scCO_2$), which has been used successfully to extract astaxanthin from *Haematococcus pluvialis* (>90-95% from the dried biomass in one step at moderate temperatures in an oxygen-free atmosphere and without residues of organic solvents remaining in the products), and
- The use of membranes to recover and fractionate bioactive molecules from a large variety of complex natural matrices.

7. ***Confirm chemical structures on selected D-Factory biological materials, including by-products of microalgal processing, establish biological activity, measure product recoveries and purities, and identify any new compounds of interest, and develop product specifications for extracts.***

Dunaliella is currently cultivated commercially for its high β-carotene content only. Biomass extracts with as yet unidentified compounds possessing anti-bacterial, anti-cancer, and other anti-oxidant activities are also known, as well as extracts with potentially high-value added pharmacologically-active compounds (eg violaxanthin, antheraxanthin, zeaxanthin, neoxanthin, phytoene, phytofluene), fatty acids and enzymes, but these have not been fully characterized or offered for commercialization.

8. ***Draw up plans for adding value to Dunaliella extracts either through formulation, or through more rigorous purification for applications in pharmaceutical, nutraceutical, food, or cosmetic industries (for new business development).***

Formulation know-how for specific high value-added products applications as well as a good understanding of current possibilities, challenges and limitations of the most important Dunaliella compounds and their properties is not available.

9. ***Establish a) conceptual designs for the construction and operation of a full-scale D-Factory demonstration facility, b) the basic functions of the high throughput platform and c) a thorough assessment of opportunities to improve materials and energy efficiency for the D-Factory demonstration facility.***

Dunaliella cells produce up to 80% of their mass as glycerol depending on biological and environmental conditions, as well as a range of carotenoids, oxycarotenoids, lipids, proteins and other compounds of commercial value. High margin compounds (pharmaceuticals etc.) require stringent purification, which is reflected in attendant higher production costs and limited market volumes, and have to be complemented with less profitable compounds such as emulsifiers and polymers, and with the lower cost margin production of, e.g. glycerol to cover basic costs. Emerging technology provides complete list of powerful, state-of-the-art systems engineering methods in process synthesis, process integration, and optimization as well as modelling environments and is ideally suited to developing an algal biorefinery.

10. ***Provide a full sustainability assessment of the technological, environmental, economic and social sustainability including a SWOT analysis on strengths, weaknesses, opportunities and threats for establishing a Dunaliella production facility and D-Factory biorefinery for the production of named compounds from Dunaliella, including the sustainable management of by-products and wastes such as salt water by month 48.***

As for many rapidly developing technologies, evaluations of single sustainability aspects, e.g. carbon footprints of algae cultivation and use have been very optimistically supporting great perspectives based on little available data. Concrete optimisation targets were however largely

unclear. This has created the need for a comprehensive sustainability analysis backed by new original data to support further development.

11. ***Build an Innovation Platform to serve as an interactive forum able to capture the interests and activities of a wide range of stakeholders and integrate their local knowledge and experience with data from the D-Factory partners so that existing knowledge can be shared and new knowledge created.***

Innovation Platforms (IPs) were introduced in 2005 as an opportunity to bring business and Government closer together to generate more innovative solutions to major policy and societal challenges. They bring together stakeholders focused on a particular challenge, and enable the integration of a range of technologies along with better coordination of policy and procurement, to deliver a step-change in performance, in the quality of public services and the ability of businesses to provide solutions for the global market place.

12. ***Develop a comprehensive business case to show industrial investors the complete opportunity and scope for the creation of a Dunaliella microalgal bio-refinery and raise additional investment for the D-Factory demonstration.***

To date, no business case for global investment in algae biorefineries and in large-scale production of microalgae using photobioreactors, algal raceways and lakes has been developed.

13. ***Manage and disseminate information at the same time as protecting the continuing acquisition of IP.***

Training workshops aimed at stakeholder management (classification, mapping for influence / importance and corresponding communication strategies) are effective tools for managing and disseminating information at the same time as protecting the continuing acquisition of IP.

Results

1. D-Factory reference library new monoclonal halotolerant microalgal strains

Twenty seven new monoclonal D-Factory *Dunaliella* strains were isolated from saline waters in Eilat, Israel and from a hypersaline salt works in Monzón, Spain, and shipped to partners for laboratory performance and characterisation. New cryogenic preservation methods were introduced and, despite the absence of a cell wall and presence of delicate flagellae, the strains were found to be well-preserved. They are now catalogued in the D-Factory reference library and available to the general public by application to the MBA Culture Collection (https://www.mba.ac.uk/facilities/culture-collection#b56).

The strains have been uniquely identified in a phylogenetic tree developed using molecular bar-coding (Fig.1), and also shown to differ in comparative studies of their growth and ability to accumulate high contents of individual carotenoids, chlorophyll and glycerol when cultivated under strictly-controlled laboratory conditions (Fig. 2).

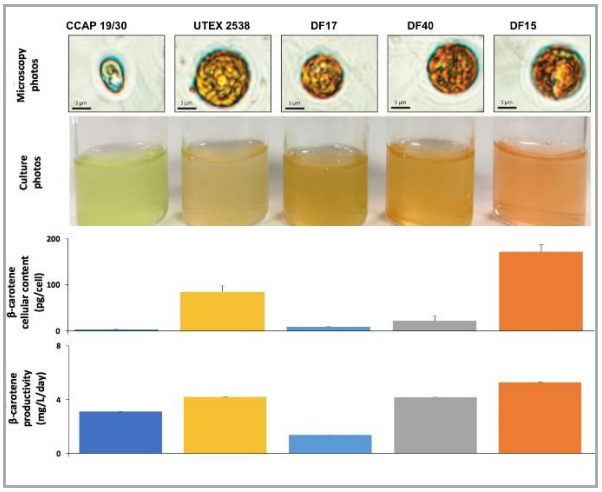


Fig. 1. Comparison of DF15, DF17 and DF40 with established D. salina strains in terms of appearance and, when grown under 1500 µmol $m^{-2}s^{-1}$ light intensity, 12h light, 12h dark cycle, productivity.

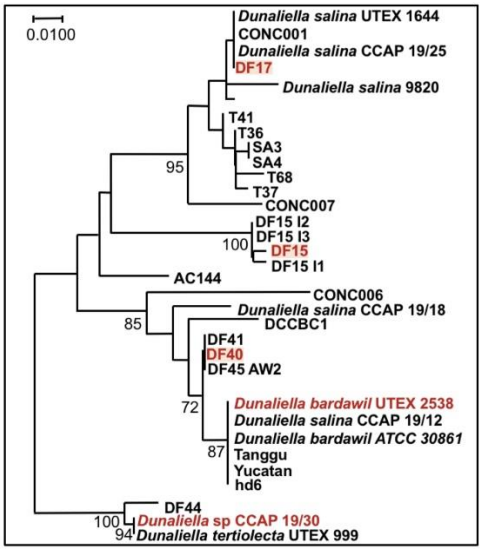


Fig. 2 Phylogenetic tree showing the location of the three newly isolated Dunaliella strains (DF15, DF17 and DF40) compared to CCAP 19/30 and UTEX 2538

D. salina strain DF40 was isolated from very high salt-crystallizing ponds in Monzón and was found to be genetically different to strains DF15 and DF17, both isolated from Eilat but surprisingly similar to *D. bardawil*, the dominant strain cultivated by NBT in Eilat. Strains DF45, DF46 & DF47, also isolated from Monzón, were shown not only to be genetically identical to each other, but also to DF40. DF40 is considered the dominant genotype in hypersaline waters in Monzón. DF40 gives a high productivity of β-carotene in highly saline water and was selected and developed for the D-Factory demonstration of Open Pond Raceway (OPR) and PBR growth methods. Strains DF44 and DF48 on the other hand were identified to be *Dunaliella tertiolecta* and *Dunaliella minuta,* respectively. Sequence data and growth characteristics for the globally-used *D. salina* standard, *CCAP19/30* suggested that it may in fact be a strain of *D. tertiolecta.* Despite having a slower growth rate DF15 from Eilat was found to have a significantly higher cellular content and higher productivity of carotenoids than DF 17, also from Eilat, and many other hyper-accumulating carotenogenic strains such as the *D. salina* (*bardawil)* strain UTEX 2538 (Fig. 1). DF15 and DF17

strains differed significantly in not only in terms of carotenoid content (carotenoid production in DF15 > DF17 at any salinity tested); but also in response to growth in ammonium (DF15 failed to grow); colour on nitrogen depletion (DF15 was orange; DF17, brown) and light intensity (DF15 bleached under very high light intensity unlike DF17). DF15 was concluded to hold substantial promise for producing large amounts of *9-cis,* and *all-trans* β-carotene, and α-carotene, even under low light intensity.

The data have been disseminated to the public and research community in conferences and the data published (open access). Details of the strains and the newly described phylogenetic tree are also the subject of Patent application numbers 1701855.7 and 1702413.4 (Algal Composition).

2. Tailoring production of halophytic algae using techniques based on strain selection and/or cultivation, in order to meet biorefinery specifications.

Laboratory systems were designed to understand how to tailor production of *Dunaliella* as a function of light, salinity, temperature and strain and evaluated in pilot-scale PBRs and open pond raceways. Laboratory mutants that were developed using a classical mutagen, 5-Bromouracil, when tested outdoors were either unable to compete with prevailing natural strains (*D. salina* mutant) or to withstand environmental conditions (*D. parva* mutant) and this approach to tailor biomass was not progressed further. Instead, effort was focused on cultivating different natural strains under different environmental conditions in the laboratory and in scale-up.

Fig. 3 Laboratory-scale PBRs for cultivating strains under strict control

From the results, the main factor influencing the amount of carotenoids produced was identified as the algal strain. All D-Factory strains of *Dunaliella* isolated by MBA were cultivated successfully at NBT in small scale from the laboratory to the medium size open ponds of 100 m^2. However, on transfer of the new DF strains to the big production open ponds of above 700 m^2, the DF strains were taken over by NBT local natural strain of *Dunaliella* and gradually disappeared, suggesting that the most practical approach for tailoring cultivation at scale would be one that was based on cultivating locally-sourced strains. Even so, when locally-sourced strains well-suited to thriving in a given locality were used, successful application of cultivation technologies for delivering specifically- tailored *Dunaliella* biomass was shown to depend on many factors. *Dunaliella* carotenogenesis is very sensitive to the light/cell ratio and also to the nitrate/cell ratio and consequently production was found to vary as a function of season, the availability and nature of nutrient nitrogen supplied, and the level of CO_2. Since carotenogenic cultures are typically stressed, they are also much more sensitive to the chemical and mineral composition of the brine and turbulence compared to non-carotenogenic cultures.

For instance, in pilot scale Green Wall (GW) and tubular PBRs at A4F, strain DF17 grew well in the green, non-carotenogenic stage, with improvements in yield afforded using non-refined sea-salt media. However culture in the orange carotenogenic stage to maximize the accumulation of carotene isomers was not readily implemented and, amongst several factors investigated, including pump speed, oxygen accumulation and light, the causes proposed included inappropriate medium chemistry, and the use of salt which was free of trace minerals. DF40 was successfully cultivated in green non-carotenogenic and also brown stage in pilot scale PBRs at A4F, but for the carotenogenic state, the same problems had to be overcome, since the strain grew well in the carotenogenic state in Monzón brine from where it had been isolated. The problems were resolved by the end of the project and growth was supported in GW (plastic and glass), tubular PBR (still with limitations), and Cascade Open Pond raceways in orange growth stage in a semi-continuous regime

for several months at A4F and Monzón. Strain DF15, one of the last strains to be tested was successfully scaled-up and cultivated in semi-continuous orange phase in GW PBRs at A4F with the improvements made.

Biomass yield increased under continuous light with non-limiting CO_2, but photosynthetic and respiratory systems were damaged. The use of continuous light is therefore not recommended for long-term continuous culture. Yields also depended on a careful balance between supply of CO_2, salinity to control predators and optimize carotenogenesis, and temperature.

For example, in the laboratory, increasing temperature (> 20^0C) increased the rate of bacterial growth and level of bacterial contamination and all laboratory-maintained cultures became increasingly cloudy, a phenomenon also observed in scale-up in PBRs. In closed flasks in the laboratory and in scale-up using tubular PBRs and Green Wall PBRs, carotenogenic cultures of cell-wall-less *Dunaliella* under stress tended to adhere to surfaces and decrease light penetration for photosynthesis. Under these conditions populations of heterotrophic microorganisms would out-compete algal populations unless their growth was controlled. Fungi could be controlled with modulation of environmental parameters including increase in salinity, but once spores germinated, measures needed to be introduced to prevent mycelial mucilage adhering to surfaces and supporting the growth of a diverse, heterotrophic, consortia. Bio-oxidants such as hypochlorite were found to be ineffective in clean-up, so the CIP routine required improvements.

Carotenoids were identified as high-value lipophilic targets for bioprocessing to meet market requirements for natural colorants and for nutraceuticals. Strong positive correlations were found between the cellular content of most of the target carotenoids i.e. *all-trans* β-carotene, *9-cis* β-carotene, *all-trans* α-carotene and zeaxanthin across all strains grown in high light stress. The ratio of these carotenoids between strains of locations is unlikely to change. However lutein was found to be a growth-coupled primary metabolite with a strong correlation with chlorophyll synthesis and respiration, therefore its content may vary as a function of growth. Ambient temperature affected carotene isomer ratios of *9-cis*: *all-trans* β-carotene. In cold temperatures *all-trans* β-carotene may precipitate; precipitation is obviated by increased production of *9-cis* β-carotene. However a novel process which increases the production of carotenes especially *9-cis*-β-carotene in all strains of *Dunaliella salina* via light quality and produces a powder or paste highly enriched in *9-cis*-β-carotene was found and developed and tested for processing performance. A patent (app no: 1719440.8 and GB1718822.8) has been applied for entitled 'Production of *Dunaliella* Process and Powder'. These developments should increase the *9-cis*-β-carotene content algal biomass at low cost and at high reproducibility and assist the profitability of β-carotene production.

In a separate development, trials using bleaching herbicides to control metabolism at the level of phytoene desaturase were successful in producing colourless **phytoene and phytofluene**. The know-how has been applied to demonstrate production at 3,000m^2.

Increased **lipid content** correlated positively with degree of nutrient or other stress.

For commodity compounds such as **glycerol**, increasing the salt concentration in the extracellular medium increased the glycerol yield, but not for extended periods of growth. Salt but not light is a significant factor controlling the production of glycerol. Cellular glycerol content also correlated with cell volume. Starch also interconverted to glycerol and vice versa depending on salt concentration in pond cultures and the rate of interconversion was rapid – within minutes.

3. Benchmarking an open pond raceway (OPR) facility producing kilogram quantities of Dunaliella biomass.

Fig. 4 Aerial view of the large industrial-scale NBT production site in Eilat, Israel

Open Pond Raceway (OPR) cultivation of *Dunaliella* as practiced by NBT Ltd in Eilat (Technology Readiness Level (TRL) =9) provided an ideal benchmark for industrial-scale production of carotenogenic biomass, against which hybrid PBR-open raceway systems could be compared as well as the production and processing activities of the D-Factory biorefinery.

In the D-Factory a pilot facility was established and, using ideal materials as well as a new cost-efficient raceway design and new techniques to control predators and reduce costs in effluent management, the productivity of the alga was doubled: NBT is now able to produce ~35 tonnes AFDW biomass per annum from 10 Ha of raceway. This facility served to benchmark OPR production of *Dunaliella* for the sustainability assessment. In spring, typical yields were ~300 mg carotene m^{-2} d^{-1}, (carotenoid : chlorophyll ratio ~10), which at ~8% AFDW carotene content amounted to approx. 4 g AFDW biomass m^{-2} d^{-1}. The cultivation site has lined raceways fitted with paddlewheels and these hold sea- and salt-water maintained at 10-15% salinity to cultivate the halophytic algae. Liquid pressurized CO_2 is bubbled in for carbonation for algal growth. Inoculation and scale-up are with smaller raceways. Algae are harvested by partially or completely draining the raceways using centrifuges and stabilised by spray-drying techniques. Raceways are operated for 300 days per year, in winter (5^0C - 15 ^{0}C) and summer (>40^0C) and wastewater containing high salt loads and organic matter is treated in aeration and settlement ponds then filtered before reuse or discharge to the sea**.** Spent water cannot be discharged to sea without strict legislative control of the BOD and COD. Predators such as Artemia are controlled with traps and with modulation of environmental parameters including increase in salinity: to allow continuous summer production NBT has to increase the salt concentration to above 18%. On the other hand, protozoa infestation is not as easily controlled; protozoa are common in all marine salts and in all ocean or marine waters. They come as cysts and cannot be eliminated. Details of the facility have been disseminated widely and were used to inform the Sustainability Assessment (see below).

Fig. 5 An open pond raceway at the demonstration site in Monzón, Spain

The knowhow to cultivate natural strains of halophytic *Dunaliella* in open pond raceways was also transferred to a second OPR facility such that it too produced kilogram quantities of *Dunaliella* biomass and was able to directly serve as a benchmark against which a hybrid PBR-open raceway system at the same locality could be compared. It also provided data for the Sustainability Assessment, namely the Monzón demo OPR in Spain, which found that sustainability of algae cultivation systems for hypersaline algae massively benefits from integration with existing salt operations and unused CO_2 sources, but integration cannot be assessed on a generic level because of very individual existing assemblages and integration options at each potential location (see details below). At Monzón, the halophytic *Dunaliella* algal strains (DF40) are cultivated in brine, which is sourced from shallow underground salt deposits infused with freshwater. During summer,

fresh water is added with the brine to balance the evaporation losses and maintain 15-25% salinity throughout the year. There are few predators because the brine comes from inland sources of salt. In the D-Factory the Monzón OPR (TRL=9) was optimized for temperature with a heating system for temperature control in winter, established with excess low grade heat from a 14MW CHP plant. OPRs were also optimized for algal CO_2 capture using flue gas CO_2 from natural gas combustion by the CHP plant (up to 7% CO_2), which is bubbled in for carbonation for algal growth. Algae are harvested in the same way as at NBT, by partially or completely draining the raceways using centrifuges then stabilised by freeze-drying techniques. Treated wastewater is injected back into empty underground salt caverns. Raceways are operated for 300 days per year, in winter (5^0C - 15 ^{0}C) and summer (>40^0C) and current biomass productivity ranges were calculated to lie between 0.75 g m^{-2} d^{-1} and 3.0 g m^{-2} d^{-1} depending on seasonality and other factors.

Fig. 6 A4F Cascade Raceway

As an additional output over and above the original project objectives, a Cascade Raceway (CRW) (capacity 15m^2, 525L) for *Dunaliella* cultivation was developed at the A4F Lisbon pilot unit and this was benchmarked as TRL=6, with average carotene productivity of ~49 mg carotene m^{-2} d^{-1}, and biomass, ~1.9 g m^{-2} d^{-1}, (carotenoid: chlorophyll ratio 7-10).

4. Development and implementation of a pilot (10 m^3) cultivation PBR

The A4F prototype unit expansion plant process flowsheet designs have centred on a high quality inoculum to be produced in GW PBRs, followed by a growth phase in tubular PBRs and finally the carotenogenesis stage for inoculation in raceways. At Lisbon a thermoregulation system was introduced which included a compression chiller and boiler for maintaining culture temperature. The A4F pilot unit expansion construction was concluded with the assembly of a 3 m^3 tubular PBR and served to provide data for the Sustainability Assessment (see details below). A pilot scale installation PBR-to-raceway process was also commissioned at Monzón, with objectives to integrate a 1000L GW and 3400L PBR from A4F for inoculation of OPR cultivation systems at Monzón in a hybrid cultivation system and provide input and output data, as well as materials data and costs of construction of the PBRs. Carotenogenesis induction by cultivation using nitrogen-limited conditions was positive and high cell densities in spring (but not summer) were recorded.

GW flat panel PBRs supported good growth of orange carotenogenic *Dunaliella* sufficient for inoculation and data collection and the plastic bags could be discarded once contaminated (TRL assessed as 9). Yields at the Monzón demo site were calculated to reach ~170 mg carotene m^{-2} d^{-1} and biomass 2.4 g m^{-2} d^{-1}, (carotenoid : chlorophyll ratio ~10).

Fig. 7. A4F-Monzon closed flat-panel GW PBR (1,000L), & tubular PBR

Tubular PBRs on the other hand were unable to sustain the growth of orange carotenogenic *Dunaliella salina* for sufficient cycles of growth to overcome issues such as internal biofilm formation (TRL 5) and therefore could not be implemented as a suitable inoculation system for open pond raceway cultivation at Monzón. The cell density in tubular PBRs reached over 3 million cells per mL in orange color. However cells were round in shape rather than the normal ellipsoid. The *Dunaliella* flagella were missing, caused by a shearing "shaving" effect, and in the first winter at Monzón, several PBR tubes were damaged due to the high temperature amplitudes, which damaged the couplings between tubes.

New polymeric-rubber couplings were introduced which provide just sufficient clamping force to avoid de-coupling and leakage. This improvement will support tubular PBR cultivations in such locations as Monzón. A further improvement would be to install tubular PBRs with glass tubing, rather than polymeric, since glass has a thermal dilation coefficient much lower than plastic. Yields were ~78 mg carotene m^{-2} d^{-1} and biomass ~2.1 g m^{-2} d^{-1}, (carotenoid: chlorophyll ratio ~8). The conclusion was that inoculation with GW-type PBR technology may confidently be used as an alternative technology to the use of mini-pond raceways, but for tubular PBRs there are still several challenges to pursue regarding carotenogenesis control, internal biofilm formation, and pumping/shearing damage to fragile *Dunaliella* cells and more data are required about consistency of cultivation results throughout consecutive seasons and years.

In summary, 3 *Dunaliella* strains were grown in 3 growth stages (green, brown and orange), in 2 regimes (batch and semi-continuous) and in 2 locations (Lisbon and Monzón) in GW flat-panel PBRs, but in tubular PBRs some challenges still exist on the path to obtain consistent growth throughout seasons and years of cultivation. This knowledge has been consolidated and disseminated to the members of the public research and community.

5. *Cell harvesting technologies to process ~2,500l saline water /h and recover at least 90% intact Dunaliella cells*

Fig. 8. Evodos T50 in situ at NBT, Eilat

Evodos spiral plate technology based on dynamic settling was implemented at three sites: (i) NBT (continuous harvesting with T50); (ii) Monzon (batch harvesting with T10) where the salinity is greater (3M) than at NBT (2M) and (iii) A4F (batch harvesting with T10). Know-how is held as confidential information within the D-Factory project although data outputs have been disseminated at https://algaebiorefineryconference.eu/wp-content/uploads/2017/11/S24.pdf. The Evodos T50 prototype harvests at 2500 Lh^{-1}, with 95% separation efficiency, >20 h d^{-1}. Pastes comprise of up to 40% solids with cells ~90% intact. However the Evodos T50 is still in development at this stage (TRL5): some component parts are under construction and critical function CIP is not yet available. Consequently the technology is not yet integrated into the process. The Evodos T10 (TRL7) harvests optimally at 250 Lh^{-1}, with 95% separation efficiency and pastes comprise of 20-30% solids with cells >95% intact. However carotene losses from harvested biomass were documented, caused by heating in the prototype T10 machine after many hours in operation and consequently the T10 is concluded as ideally suited for small-scale operations with very high density cultures (>1x10^6 cells mL^{-1}).

Nevertheless in a trial to harvest *Dunaliella* from the same pond at Monzón and compare harvesting efficacy between Evodos- or Westfalia-type (disc-stack, TRL9) harvesting systems, substantial losses of biomass were identified with both machines. This was improved by diluting the salt concentration of Monzón cultivation ponds. Evodos-harvesting technology was slow compared to Westfalia, and under high ambient temperatures, the concentration of susceptible carotenoid isomers was reduced. Evodos-harvested powder also behaved differently to Westfalia-harvested powder and this currently impacts in processing with $scCO_2$. Nevertheless Evodos technology permits recovery of cytosolic components for improved product recovery and may reduce effluent treatment.

An ultrafiltration polysulfone membrane unit (membrane area of 6.1 m^2; cut-off MWCO of 100 kDa) was also tested at the Monzon demonstration site to harvest carotenogenic cells intact using controlled permeate flux conditions with mild cross-flow velocity of around 17 - 22L m^{-2} h^{-1} and low transmembrane pressure, achieved by eliminating a control valve in the retentate side. Wash cycles of 9 min permeation + 1 min relaxation were used at a feed cross-flow velocity of 0.30 m/s (corresponding to a feed Reynolds number Refeed of 284) to minimise membrane fouling. At up to 5-fold concentration cells were immobile, round instead of pear-shaped and without flagellae, but appeared intact, and lipophilic carotenoids in cell membranes were retained. At more than 5-fold concentration cells were visibly damaged and at 10-fold concentration although carotenoids and chlorophyll were retained, at least 60% glycerol was lost to permeate. The technology is not yet integrated into the process or tested for scale-up (TRL= 4/5) and is slow, at 0.1 m^3 h^{-1} per unit compared to a disc-stack centrifuge processing 10 m^3 h^{-1}. Consequently in-line processing to match current productivity would require 100 membrane units followed by a post-membrane centrifugation to concentrate from estimated 2% w/w paste to above 10% w/w paste. Nevertheless an economic evaluation showed that the harvesting of *Dunaliella salina* using a two-step approach integrating membrane processing with Evodos spiral-plate centrifugation is to be preferred over using solely centrifugation in terms of capital and operational costs. Based on a concentration factor of 5 and an average permeate flux of 22L/(m^2.h), a reduction of the OPEX + CAPEX of 52% and reduction of energy consumption of 66% can be achieved compared to only harvesting by Evodos spiral-plate centrifugation. This conclusion was made even without considering the permeate recycle as cultivation medium, which represents additional significant savings when using the two-step approach. The data have been published in Separation and Purification Technology 190 (2018) 252-260 and Algal Research 24 (2017) 325–332.

Fig. 9. Membrane unit at Monzón

Effluent/spent culture medium from cultivation and harvesting currently requires intensive treatment to meet current legislation. A combination of UV and H_2O_2 treatment of permeate obtained after concentration of intact cells from spent culture medium using membranes was tested in 1.5L batches and shown to degrade organic matter such as glycerol, whilst oxidation by-products such as nitrate and nitrite did not accumulate. The treated effluent was tested for recycle; culture medium recipe modifications at A4F enabled better performance in the carotenogenesis trials with all strains, but further work can be done, namely in fine-tuning of nitrate/cell ratio, [Mg] and trace elements, to achieve higher productivity values.

6. Storage, handling, disruption and bioprocessing to produce extracts/fractions for bio/chemical profiling and bioactivity screening, and for D-Factory scale-up designs to industrial scale, taking into account technical, environmental and economic aspects using data obtained from mobile units installed at the D-Factory demonstration production site.

Delivering maximal value from biomass with minimal waste underpins the concept of a modern biorefinery. From literature and market research, several so-called ‘high-value’ targets were identified for developing the D-Factory biorefinery using *Dunaliella salina* biomass:

- Lipophilic antioxidant compounds such as carotenoids and unsaturated fatty acids, with particular value as nutraceuticals and possibly cosmetics, and
- Water-soluble enzymes with value in clinical diagnostics.

However two problems were encountered in the course of initial attempts to deliver both sets of targets - water-soluble enzymes and lipophilic compounds - from harvested biomass. First,

processing with aqueous solvents to extract water-soluble enzymes had a negative impact on the recovery of valuable carotenoids. Second, stabilising harvested biomass either with alkali, or as a spray-dried powder to preserve carotenoids, destroyed enzyme activity. The focus therefore shifted to concentrate on processing biomass for lipophilic targets and a high-level processing schematic was developed in order to assess whether it would be feasible to isolate lipophilic targets and also account for all harvested algal biomass.

Sample preparation for shipping: Samples needed to be shipped to partner sites for processing. The proposed technological innovations based on the use of large supercritical CO_2 units for industrial-scale processing, which require significant CAPEX investment, and HPCCC both needed to be evaluated for sustainability before adoption. This would help to anticipate any unwanted effects or potential damage.

Wet algal biomass was found to be highly susceptible to deterioration after harvest and consequently a strictly controlled method for shipping freshly-harvested wet cells from production sites to processing sites was established. This was especially important for shipping intact cells because freeze-thaw in transit caused fragile algal cells to burst but fungal mycelia and bacterial cells remained intact and as heterotrophs, the latter quickly colonized disrupted algal cells. A solution of 30%wt glycerol in water was shown to be a suitable preservative of fresh harvested cells, however since algal pastes contain heterotrophic bacteria they are best processed as rapidly as possible. Powder samples of biomass or of extracts that had been dried to remove water were readily shipped to partners in a stable condition, provided they were also held in the dark and shipped under vacuum in sealed containers or stored in a desiccator under argon or nitrogen at temperatures below -20^{o}C to prevent degradation. This latter precaution was found to be especially important in samples containing hygroscopic glycerol, which also lowered the freezing point of samples. Shipped as dried powders, samples could be sourced for method development from any of three locations (NBT, Monzon and A4F) and harvested as either intact, unwashed cells (Evodos harvesting method) or as cell-disrupted, washed algal biomass (Westfalia method) and dried by either spray dry or lyophilisation. Also extracting wet (previously frozen) pastes with solvents was found to be ineffectual in extracting carotenoids and resulted in thick emulsions which would not filter or partition. Consequently samples were routinely shipped as dried powders. However dryers are either spray-driers or lyophilisers, and these are not equivalent: Spray-drying involves use of a hot drying gas, which can denature enzymes and produces a fine (100-300μm) free-flowing powder. This is suitable for processing with chemical petroleum solvents but nevertheless still needs improvements for processing with scCO2, whereas lyophilisers use a combination of reduced pressure and enough heat for ice to sublime from pre-frozen material and the resultant powders are well-suited to $scCO_2$ processing. Consequently only lyophilised cell powders supplied by Monzón Biotech were used for $scCO_2$ processing.

Washing: Washing Westfalia-harvested biomass free of salt resulted in some losses of organic matter, including polar carotenoids and also reduced the content of 9-*cis* β-carotene relative to *all-trans* β-carotene: the former is known to be labile and easily destroyed. Washing also reduced the relative content (AFDW) of polar carotenoids and chlorophyll derivatives. At NBT a method was developed such that washing is without effect on recovery of carotenoids and best practice has been transferred.

Biomass disruption: Biomass disruption before extraction with solvents for polar lipids or proteins was found to be essential; ultrasonication was the most efficient laboratory scale method, but also extractions using organic solvents as well as freeze thaw cycles were also effective. The conditions used for ultrasonication needs to be adjusted to fit the purpose of the material to be extracted.

Extraction using supercritical CO_2: The use of supercritical CO_2 with lyophilised powders of *Dunaliella* yielded enriched extracts of high-value carotenoids for formulation testing and defatted powders for by-product development. Conditions for processing Westfalia-harvested *Dunaliella* biomass with $scCO_2$ were optimised (TRL=9) and production of carotenoid extract and defatted powder of the quality currently required by the market is now available from NATECO on contract; annually a minimum of 100 t of biomass can be processed in 185 working days. The oily extract typically represented 16-17% by weight of total algal biomass. Around 30 % of this extract was made up of carotenoids, of which >87% are hydrophobic carotenes. However ~50% of the oil still remains in the defatted powder, and includes xanthophylls. Linolenic acid (18:3), linoleic acid (18:2), oleic acid (18:1) and palmitic acid (16:0) were the predominant fatty acids in both defatted powder and extract.

Fig. 10. Freeze-dried Westfalia-harvested biomass shipped in batches under vacuum for processing with $scCO_2$

Fig. 11. Industrial plant HD12 for scCO2-extraction of algae biomass at

NATECO also carried out extraction of Evodos-harvested *Dunaliella* biomass with $scCO_2$: the conditions have not been optimised but from preliminary data there was a loss of carotenoids and total extracts such that the yield of extract was 2 % and therefore considerably lower compared to powders prepared using Westfalia-harvested biomass and extracted with the same parameters.

Extraction using petrochemical solvents*:* Use of petrochemical solvents was bench-marked against use of $scCO_2$. Polar and non-polar combinations of solvent (methanol or acetone followed by hexane; (MTBE-methanol) will extract almost all carotenoids present in biomass (91%), but extracts are not as enriched in carotenoids as for $scCO_2$ extracts. Extracts prepared using petrochemical solvents typically represented ~ 35% by weight of the total material and contained ~14% total carotenoids.

Further downstream processing: Laboratory-scale investigation indicated that carotene-enriched extracts from either $scCO_2$ extraction or solvent extraction were highly suited for further processing using further combinations of solvent fractionation, counter current chromatography (HPCCC) and preparative HPLC technologies, with the use of solvent resistant membranes to remove solvent. An integrated purification strategy for high –value carotenoids was therefore established and then tested with the aim of scale-up (Fig. 12).

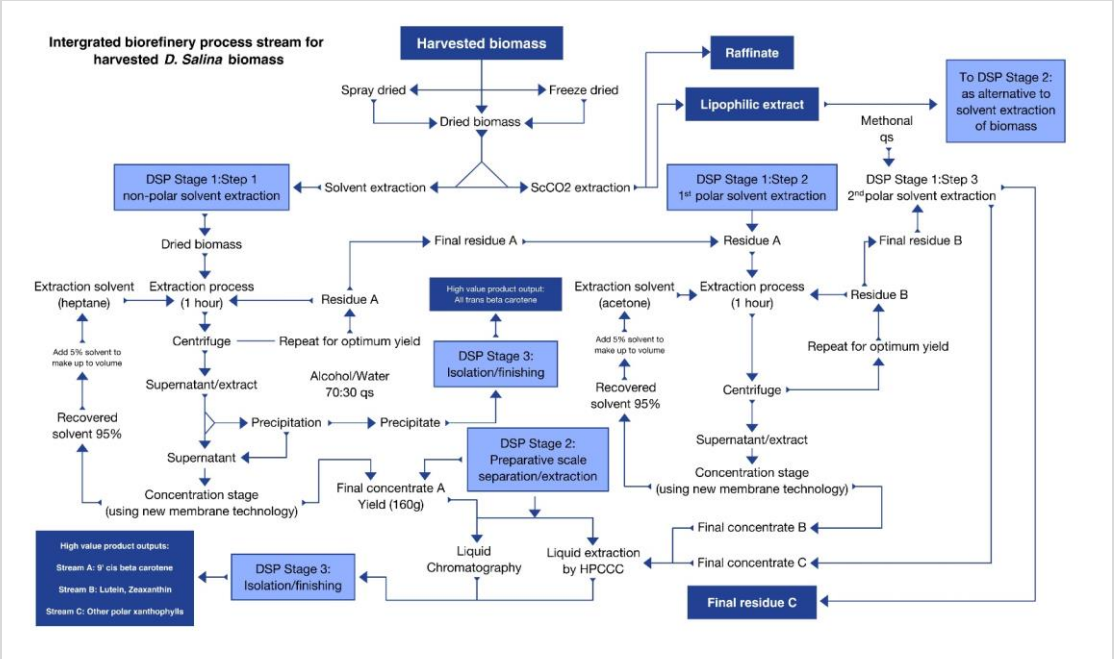


Fig. 12. Integrated biorefinery process stream for harvested D. salina powders

However the chemistries (size, polarity) of carotene isomers are very similar; the chemistries of lutein and zeaxanthin are also very similar and share features in common with pheophorbides. These problems are easily solved in analytical and laboratory-scale separations (Fig.13) but extremely challenging in scale-up (Fig.14). The purified carotenes are also unstable to light and oxygen and increasingly unstable on purification from lipids.

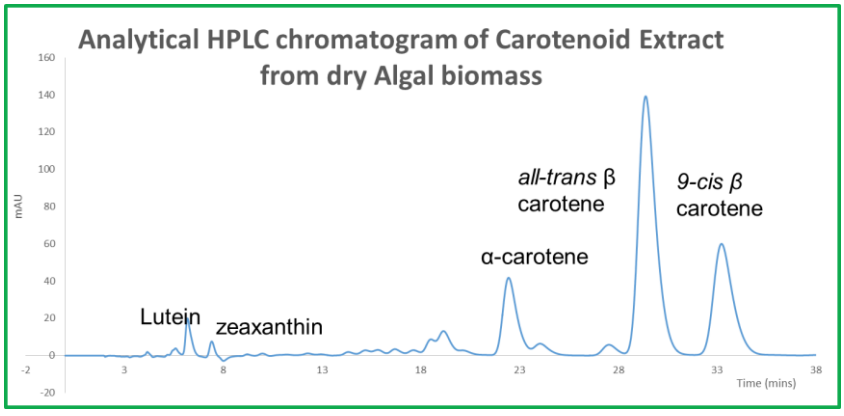


Fig. 13. Carotenoid isomers were well-resolved with analytical HPLC. Analytical HPLC was performed using a YMC30 250 X 4.9mm I.D S-5µ HPLC column with DAD at 25C; Isocratic elution with 80% methanol: 20% MTBE, Flow 1 mL/min. 450nm profile shown.

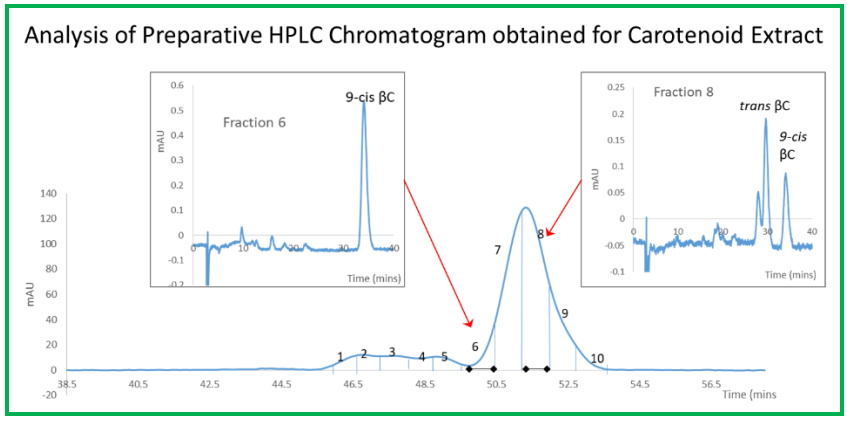


Fig. 14. Carotenoid isomers were poorly resolved using preparative HPLC. Preparative HPLC was performed using a C18 HPLC column with DAD at 25C; Isocratic elution with 95% methanol: 5% acetone. Inserts show analysis of Fractions 6 and 8, conditions as for analytical HPLC

The carotene isomers**,** *9-cis*-beta carotene, *all-trans* β-carotene, α-carotene and others, could not be separated using HPCCC due to their similar partition coefficient in the range of solvents systems tested. Instead, a strategy using HPLC was developed.

Preparative HPLC is a mature technology (TRL8) but successful separation depends on the nature of the starting material. Using *Dunaliella* $scCO_2$ or solvent carotenoid extracts, efforts to separate the carotenoid isomers were characterized by low product yields, long run times and low loading capacity (Fig.14). However using enriched carotene preparations greatly improved HPLC resolution between isomers and these were obtained by incorporating a processing step with either anti-solvent or temperature-dependent methods to precipitate *all-trans* β-carotene and preferentially retain *9-cis* β-carotene in solution. Good recovery of *9-cis* β-carotene (>70%) was possible with a separation efficacy of *all-trans* β-carotene of ~80%. Using semi-preparative HPLC on these preparations delivered samples of *9-cis* β-carotene with only ~18% other material (likely lipid) and which represented 94% total carotenoids. However only 40% yield was achieved. At pilot scale, the best preparative sample of *9-cis* β-carotene contained ~83% other material (likely lipid), even though the sample was ~90% of total carotenoids. The ratio of 9cis*: all-trans* β-carotene increased to 90.8. 9-*cis* β-carotene is more susceptible to oxidation than *all-trans* β-carotene and therefore enrichment may favour auto-oxidation with time. Cooling (freezing) was assessed as being able to reduce CAPEX substantially compared to the use of anti-solvent in the precipitation step to enrich *9-cis* β-carotene separately from *all-trans* β-carotene, but HPLC was concluded not to be a cost-effective choice for these types of difficult separation. A cost-effective, sustainable, high-efficiency and low environmental impact commercial process to purify 9-*cis*-β-carotene from *all-trans* β-carotene remains to be developed.

The application of HPCCC technologies to process *Dunaliella* materials and deliver enriched preparations of xanthophylls was demonstrated.

Fig 15. Images of the modular HPCCC unit. Top L: computer image showing a bank of units, which are illustrated in different orientations in the 3 other images.

For scale-up, a prototype processor with a column volume of 6000ml had originally been intended as the large scale commercial operating unit but the designs were assessed as being cost-inefficient and unlikely to be sustainable. Instead a modular system (Fig. 15) was designed and a new process based on this system and suitable for industrial application was developed (Patent application 1718939.0 filed 16 November). However the chemistries of the single unit still need further development because they were found to be insufficient to separate contaminating chlorophylls and pheophorbides from the *Dunaliella* pigments and require further development to deliver enriched preparations of individual carotenoids especially *9-cis* β-, *all-trans*-β- and α-carotene isomers, lutein and zeaxanthin, as well as chlorophyll, polar lipid, and non-polar lipid (TRL2-3). Organic Solvent Nanofiltration (Duramem 150, at 20ºC and 30 bar) concentrated successfully a preparative HPLC fraction of carotene in a mixture of methanol:acetone (19:1 v/v) with 80% of solvent recovery (which could be higher, if required) and 100% of carotenoids rejection.

By-products and wastes: From a systematic review of D-Factory processes associated with the cultivation of *Dunaliella* in hypersaline water and subsequent processing to products the main source of waste was found to arise from biomass released from cells that were ruptured in the course of harvesting using Westfalia-type disc stack centrifuges. Conversely harvesting cells intact using Evodos spiral plate centrifugation offered the opportunity to recover enzyme and polar lipopolysaccharide by-products economically from cell biomass. The advantages of harvesting cells intact are two-fold: additional products to augment the D-Factory portfolio, which is currently based

on lipophilic carotenoids and lipids and defatted powder, and reduction in costs of effluent waste management. Successfully implemented, harvesting cells intact would support the subsequent implementation of relatively simple processes e.g. controlled cell rupture with water coupled to centrifugation such that all algal biomass may be accounted for and a portfolio of principle products and co-products, with minimal waste, delivered. Downstream processing with supercritical CO_2 will generate a small loss of CO_2, because most is recovered by condensation and re-used in the process, but there are no further wastes. Wastes from downstream processing with solvents can be minimised by implementing solvent recycling combined with the use of membranes to recover solvents.

7. ***Detailed biochemical work: chemical structures of selected D-Factory biological materials, including by-products of microalgal processing, biological activity, product recoveries and purities, new compounds of interest, and product specifications for extracts.***

Analytical protocols were defined for quantifying protein, carbohydrate, lipid, and ash as well as pigments and carotenoids in carotenoid extracts and defatted powder. Fast lipid, chlorophyll and carotenoid quantification using APCI-MS and analysis with UPCC with PDA-ELSD-MS detectors in-line were applied to evaluate the nature of compounds in enriched preparations and a UV-vis spectroscopy method was developed and optimized to assess the degradation of carotenoids in different water continuous formulations. NMR methods were established to confirm structures of isolated compounds and bioassays were designed to test bio-activity of individual fractions from biomass. A methodology was developed to study carotenoid penetration into the skin using confocal Raman spectroscopy.

Products and product specifications: Multiple sets of data were obtained from analysis of *Dunaliella salina* powders that had been prepared from cultivation of the alga at two sites, NBT, Israel and the facility at Monzon, Spain and analysis of all potential products, by-products/co-products, bioactive products and wastes obtained after processing. From these, detailed specifications of the D-Factory products were developed (See Fig. 16). Several of these specifications are published in a brochure available at https://www.d-factoryalgae.eu/profiles/dfactory/images/file/brochure_dfactory.pdf .

Carotenoid extracts and the products (carotenes, xanthophylls and their isomers) derived from them are expected to find growing favour as natural products in global markets with health benefits as food supplements or as colorants. *Dunaliella salina* is listed as having no toxins known and GRAS; carotene extract from *Dunaliella salina* has been evaluated positively as a food additive and EFSA has concluded mixed β-carotenes obtained from algae as food colour is not of safety concern. They will enter cosmeceutical and nutraceutical markets, especially after registration on REACH and approval of a medicine licence after clinical trials respectively.

From lipid composition of powders, a relatively high concentration of PUFAs are present, which have important nutritional value, especially α-linolenic acid (C18:3n-3) (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which cannot be synthesised by animals, unless ALA is provided as a precursor. These will be retained in carotenoid-enriched extracts of powders, providing added value.

Proteins are retained in defatted powders and, from amino acid analysis of the powders, histidine was determined as the limiting essential amino acid for both chick feed and human food in Evodos-harvested powders where cells are harvested intact, and lysine in Westfalia-harvested powders where cells are ruptured to release cytosolic contents.

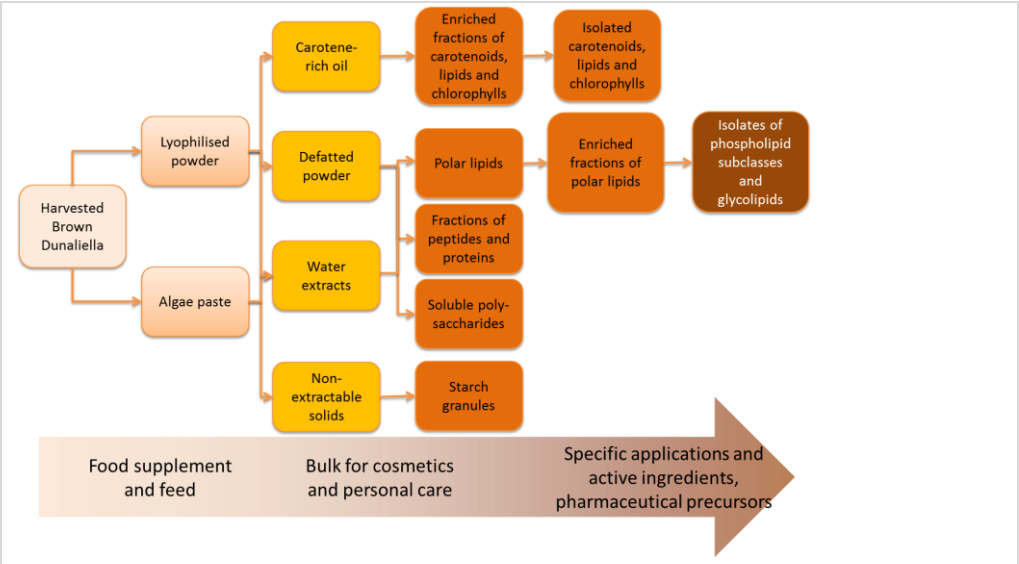


Fig. 16. Overview of products and extracts developed in the D-Factory project from Dunaliella salina.

Sources of biomass variability: Detailed biochemical work also identified that the greatest source of variability in powder composition arose from the manner by which biomass is harvested. In biomass prepared using Evodos technology at either Monzón, where strains related to DF40 predominate, or from NBT (strains related to DF15 and *D. bardawil*) cells are harvested largely intact, and are dried without washing. Biomass prepared using Westfalia technology ruptures cells during harvest and includes a washing step to remove salt on site. These findings held true for algal powders sourced across different seasons and different years. From laboratory studies the time of harvesting within the 24h cycle was also found to impact on the composition of cell carbohydrates (glycerol or starch).

Detailed analysis of purified compounds: Further examples of biochemical analysis include realisation that lutein preparations from HPCCC that were deemed to contain primarily lutein with trace amounts of zeaxanthin (14 parts lutein: 1 part zeaxanthin) using HPLC, also contained **adonixanthin.** The presence of this compound was identified using the powerful analytical capability of UPCC with PDA-ELSD-MS detectors in-line.

Purified preparations of the isomer **9-*cis* β-carotene** were shown to represent 94% of total carotenoids using HPLC with a PDA detector, but only 82% w/w of the dry matter. Analysis with UPCC with PDA-ELSD-MS detectors in-line identified the contaminants as different **fatty acids** (See Fig. 17). These fatty acids/lipids may associate with *9-cis β*-carotene molecules and contribute to formation of a super-structure in the presence of water. Lipids may also mask the absorbance of carotenes and modify their extinction coefficients. Using these techniques, enriched samples of *9-cis* β-carotene were also tested for stability and shown to be stable for up to 3 months at 4°C in petrochemical solvents but degraded readily in the presence of water containing oxygen and light. They were stable once evaporated to dryness with an inert gas and stored at low temperature.

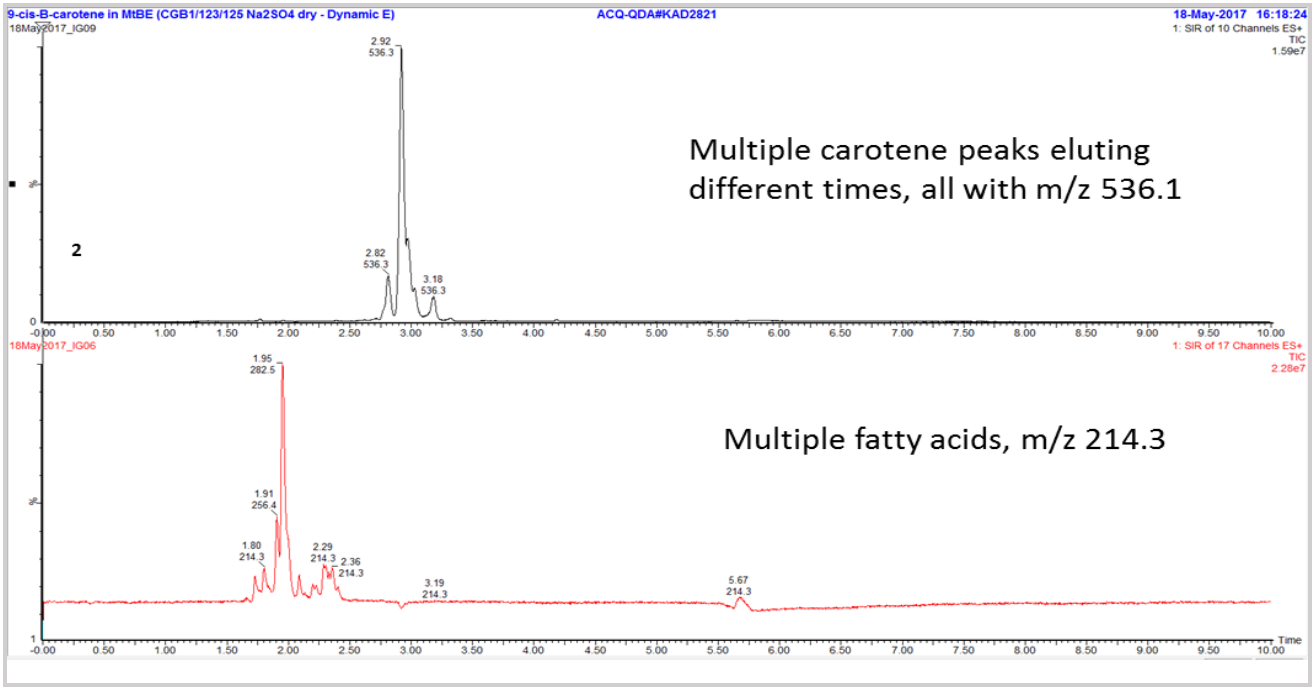


Fig. 17 Analysis of 9-cis-β-carotene using UPCC with PDA-ELSD-MS detectors in-line

Bioactivity screening: Samples for bioactivity screening included spray-dried or freeze-dried algae powders; pastes from freshly harvested algae; algae harvested with either a disc-stack centrifuge to provide lipidic fractions enriched with carotenoids or using Evodos spiral plate technology, to provide intact algal cells, and different strains of algae e.g. DF40 from Monzon; DF15 from Israel. Tested fractions comprised mixtures of different compounds obtained using non-aqueous solvents including methanol, acetone and hexane. Bioactive fractions could therefore be classified as solvent-resistant and either polar or non-polar. Some of the source materials had been subjected to heat denaturation (e.g. NBT powders) and could therefore be classified as heat-resistant molecules as well as solvent-resistant. Samples were also obtained after fractionation from acetone extracts using HPCCC or using $scCO_2$ and fractionation of extracts for peptide extracts. Fractions were characterized according to elution behavior after reverse-phase (C18 or C30) chromatography and absorbance spectrum obtained at 450 nm. No single pure isolated compound was tested for bioactivity.

Evidence for cytotoxic activity, anti-oxidant activity, anti-inflammatory activity, anti-bacterial activity and anti-diabetic activity was detected in all preparations except those prepared specifically for peptides: no bioactivity was associated with extracted peptides. Furthermore anti-oxidant enzyme activity was only detected in fresh preparations of cells prepared using aqueous buffers.

Polar active agents with anti-diabetic activity measured as anti-α-glucosidase activity were concentrated in lipidic membranous pelleted material but are not carotenoids. Active agents with cytotoxic activity were solvent- and heat-resistant, and not linked to carotenoid function. Active agents with antibacterial activity were tentatively linked to carotenoid function. Antioxidant activity was associated with carotenoid content especially *9-cis* β-carotene and was detected in all fresh strains of algae tested and in powders supplied by NBT. Antioxidants such as lutein, β-carotene and polyunsaturated fatty acids are known to have anti-inflammatory effects. Anti-oxidant activity was also associated with the enzymes catalase, peroxidase and superoxide dismutase and strains varied in the respective contents of these enzymes.

8. Plans for adding value to Dunaliella extracts either through formulation, or through more rigorous purification for applications in pharmaceutical, nutraceutical, food, or cosmetic industries (for new business development).

There is strong interest in bio-based algal carotenoids with anti-oxidant properties to replace synthetic carotenoids in food, feed, nutraceutical additives and cosmetics. In skin applications for example, topical β-carotene that has penetrated well into human epidermis is converted into retinyl esters by human epidermis and thus appears as a precursor of epidermal vitamin A. It has importance in the maintenance of healthy epithelium, and topical delivery of retinoids partly counteracts UVB-induced vitamin A depletion and promotes skin recovery. To this end the formation of a range of emulsions and powders were developed for further applications by stakeholders. These are described in https://www.d-factoryalgae.eu/profiles/dfactory/images/file/D-Factory_Application-Note.pdf and have been disseminated widely to stimulate stakeholder engagement

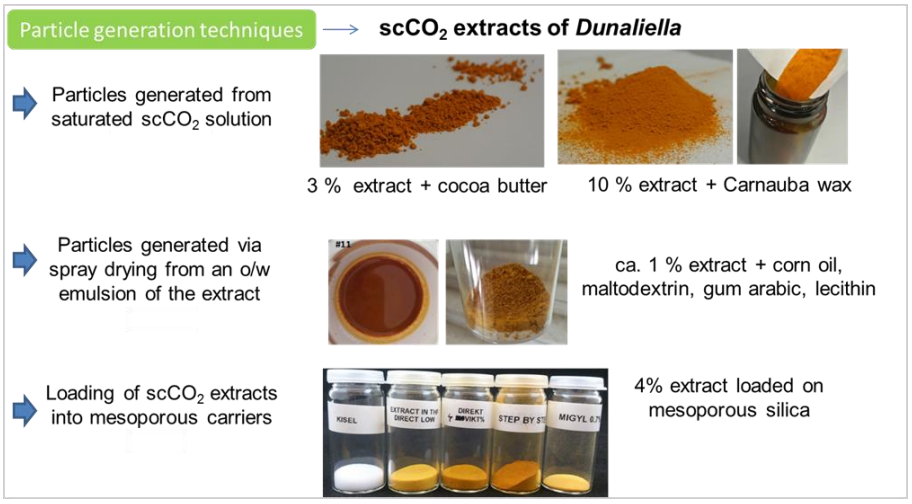


Fig. 18: Overview of different technologies and approaches tested for the generation of carrier materials for the carotenoid-rich scCO2 extracts in powder form

Transformation of carotenoid extracts to powders using PGSS: A unit to generate a powdery material from carotenoid-rich $scCO_2$ extracts obtained from *Dunaliella salina* powders by transforming these using a technique called PGSS (particles from gas- saturated $scCO_2$) was installed and tested. Work on various options for stable presentation of extracts resulted in several stable dry products as well as the laboratory production of some food products which found initial acceptance in trials.

The carotenoid-rich $scCO_2$ extract was also incorporated into water continuous nanoemulsions or lipid nanodispersions as prototype systems for oral and topical delivery of carotenoids. Increasing the overall carotenoid concentration in the emulsion through an increase in the amount of dispersed phase produced a significant reduction of the degradation rate of the carotenoids in the nanoemulsion systems.

Isolation of granular starch: A method was developed and optimized for the isolation of granular starch from the spent biomass obtained after $scCO_2$ extraction and dried to a free-flowing powder for the stabilisation of o/w emulsions. This starch displayed similar characteristics to starch isolated from spray-dried *Dunaliella* biomass in terms of its crystalline character and functional properties as a thickener.

The small size (ca. 1.5 - 3 µm median diameter, Fig. 19) makes them an ideal candidate for the preparation of highly robust, edible, particle stabilised (Pickering) emulsions. However efforts to stabilise oil-water emulsions using the *Dunaliella* granules have not yet been successful because they were resistant to hydrophobic modification using conventional methods eg through reaction with Octenyl Succinic Anhydride. Tuning the modification protocol to induce a greater extent of hydrophobicity in the granules is expected to greatly enhance the stability of the resulting emulsions.

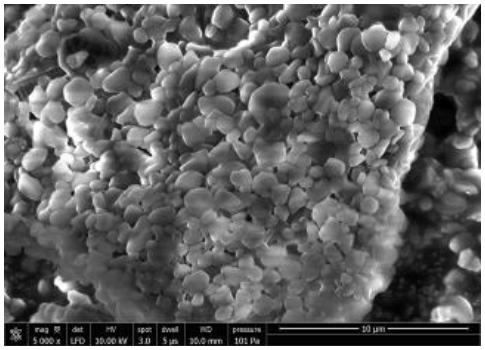


Fig. 19. SEM micrographs of starch isolated from defatted Dunaliella powder (after freeze drying). Scale bar: 10 µm

Spray drying encapsulation: Process conditions were optimised for preparing particles comprising a 3% $scCO_2$ extract of carotenoids emulsified and encapsulated in 37% soya lecithin, and olive oil (50:50 % wt) all in a solution of maltodextrin and gum Arabic. The powder carotenoid content and the carotenoid encapsulation efficiency of the process decreased with scale up. Nevertheless the carotenoids encapsulation efficiency of the semi pilot scale process was 34% corresponding to a powder carotenoid content of 2%.

Emulsifying properties of polar lipids: A methodology was developed for assessing the emulsifying properties of polar lipids isolated from *Dunaliella salina* (freeze dried powder). The polar lipid fraction analysed (rich in phosphoserine and phosphocholine) favoured the formation of o/w emulsions with a limited range of oils with respect to soy lecithin. Polar lipid fractions with hydrophilic-lipophilic balance (HLB) ranged from 14 to 5 depending on the nature of biomass used.

New food products: Defatted *Dunaliella* biomass was successfully demonstrated as source of protein and starch in fish sausages. Fish sausages containing 2.3% and 4.7% defatted biomass had a darker colour and a neutral, but pleasant taste. The best overall results (appearance, texture and taste) were obtained in a fish sausage containing 2.3% of defatted biomass.

Fig. 20. Fish sausages made with D. salina defatted powder

Fig. 21. Albumin from defatted powder incorporated into bread

The manufacture of appetizing looking, gluten-free bread, with a good structure was also demonstrated using the *Dunaliella* Albumin protein fraction.

Feed has now been produced using $scCO_2$ extraction for trials using aged mice and fruit flies (carotenoid extracts) and chicks (defatted powder). In a 1000-chick feed study, a low additive inclusion levels of *D. salina* defatted powder displayed bioactive properties when fed to male broiler chickens. These beneficial effects were depressed and negative responses were observed at higher ingredient levels suggesting a product which might be used at low dose to help in the drive to find alternatives to antibiotics (now banned) in animal feeds. These data laid the foundations for the D-Factory business case.

A brochure and application note of D-Factory products were produced and are available at https://www.d-factoryalgae.eu/profiles/dfactory/images/file/brochure_dfactory.pdf and https://www.d-factoryalgae.eu/profiles/dfactory/images/file/D-Factory_Application-Note.pdf

9. ***Establish a) conceptual designs for the construction and operation of a full-scale D-Factory demonstration facility, b) the basic functions of the high throughput platform and c) a thorough assessment of opportunities to improve materials and energy efficiency for the D-Factory demonstration facility.***

Integrated designs were established for construction of a fully operational industrial scale D-Factory facility in year 2025 after close collaboration between the modelling and experimental groups, groups involved with sustainability and groups devoted to pilot development. Several potential plans were proposed and studied to introduce a significant advancement in the state of the art in the form of scenarios (See Fig. 22).

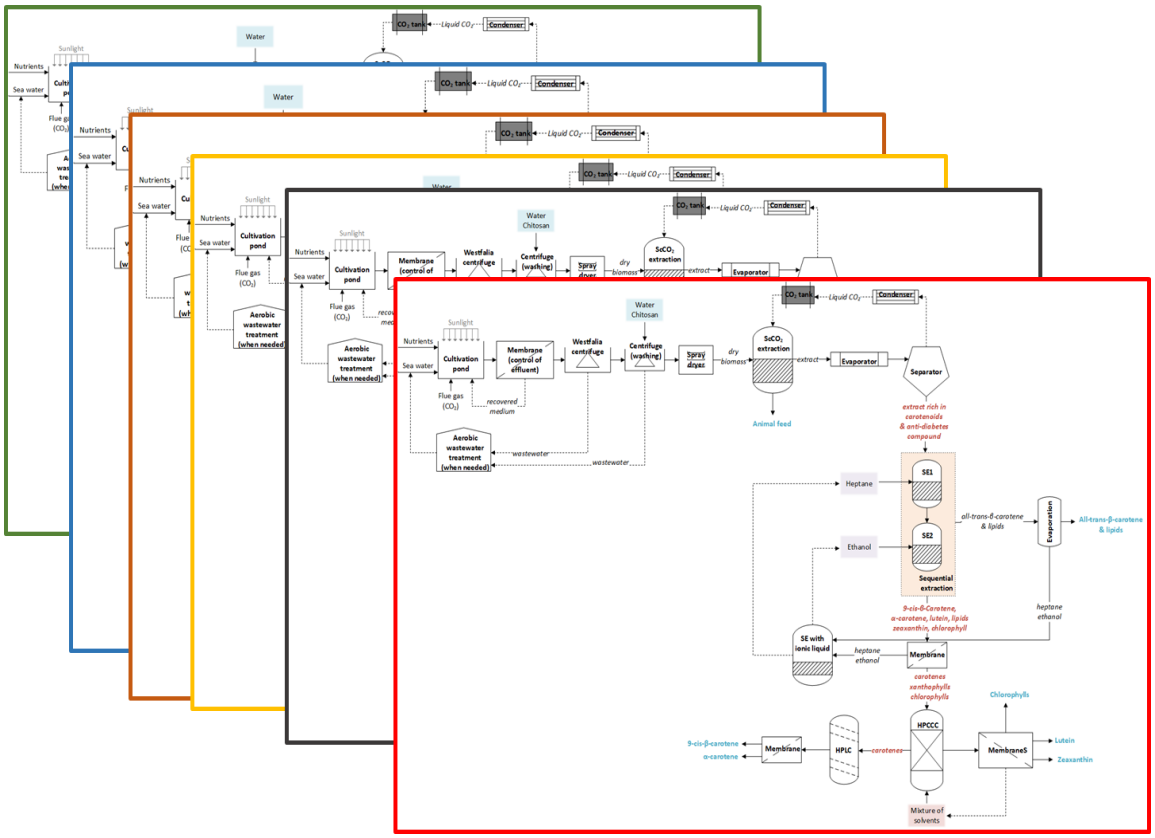


Fig. 22. Conceptual designs developed for a full integrated sustainability assessment – costs, LCA, social, technical – of a full-scale D-Factory demonstration facility

These were integrated using simulation software. The design methodology conformed to a product-oriented approach, according to which the products were established, and the process selection followed.

Carotenoids, lipids and glycerol comprised the product portfolio, whereas centrifuges, supercritical fluid extraction, solvent extraction, liquid-liquid extraction, membranes and chromatography were the main involved technologies.

The D-Factory platform has been developed and constitutes a powerful modelling tool, which dynamically builds and graphically represents value chains and processing paths from algal strains. It is built upon Yii framework, a high-performance PHP framework for creating Web 2.0 applications and exploits semantics and ontology engineering to express the system flows, process components and their relationships and finally to represent the synthesis pathways. An ontology which

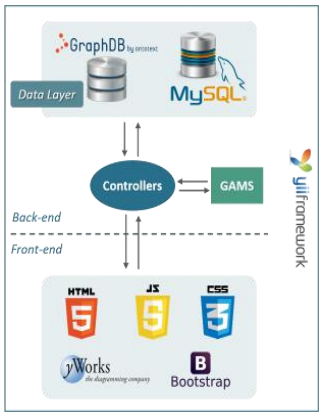


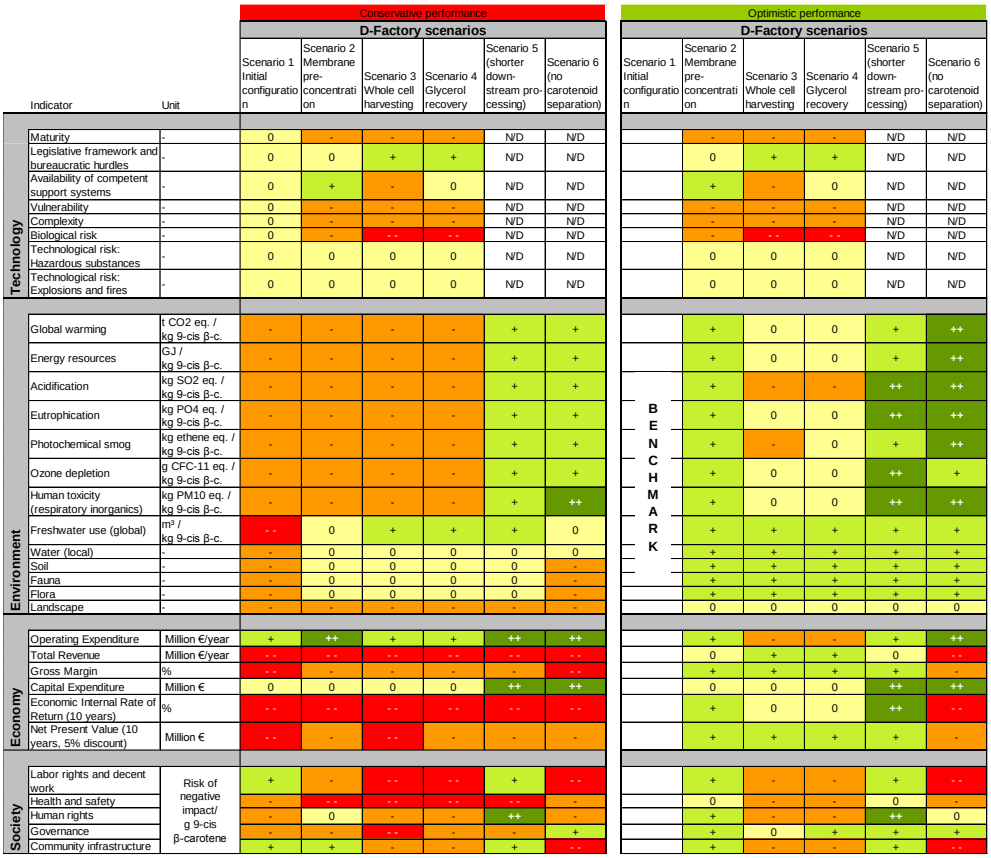
Fig 23. D-Platform architecture

formulates the value chain of an algae biorefinery has been designed using Protégé, a free, open-source ontology editor and framework for building intelligent systems. The platform sets a benchmark on a wide range of D-Factory products and pathways by evaluating a set of case studies. In this way, it includes data from all partners involved in the project, thus providing an integrated database of all the stages of microalgae biorefineries. Fig. 23 illustrates the Model-View-Controller (MVC) software design pattern used for the Platform.

Energy data were screened concerning individual units and incorporating processes of auxiliary operation focusing on future feasible microalgae biorefineries.

10 Full sustainability assessment of the technological, environmental, economic and social sustainability including a SWOT analysis on strengths, weaknesses, opportunities and threats for establishing a Dunaliella production facility and D-Factory biorefinery for the production of named compounds from Dunaliella, including the sustainable management of by-products and wastes such as salt water

A list of 14 products and by-products/co-products was derived after extensive analyses and an integrated sustainability assessment led by IFEU analysed the sustainability impacts of the newly devised processes to deliver these products. It joins detailed analyses of technological, environmental, economic and social aspects (by UoG [Harvey 2017], IFEU [Keller, Gärtner, et al. 2017], HI [Mitchell & Goacher 2017], and RISE [Peñaloza & Stahl 2017], respectively) into an overall picture and derives common conclusions and recommendations [Keller, Reinhardt, et al. 2017]. It is based on scenarios for 2025 to support decisions to be made during the implementation process. Please refer to these public reports for additional insights and recommendations exceeding this summary, results and methodology.



	Indicator	Unit	Conservative performance: Scenario 1 Initial configuration	Scenario 2 Membrane pre-concentration	Scenario 3 Whole cell harvesting	Scenario 4 Glycerol recovery	Scenario 5 (shorter down-stream pro-cessing)	Scenario 6 (no carotenoid separation)	Optimistic performance: Scenario 1 Initial configuration	Scenario 2 Membrane pre-concentration	Scenario 3 Whole cell harvesting	Scenario 4 Glycerol recovery	Scenario 5 (shorter down-stream pro-cessing)	Scenario 6 (no carotenoid separation)
			D-Factory scenarios						D-Factory scenarios					
Technology	Maturity	-	0	-	-	-	N/D	N/D		-	-	-	N/D	N/D
	Legislative framework and bureaucratic hurdles	-	0	0	+	+	N/D	N/D		0	+	+	N/D	N/D
	Availability of competent support systems	-	0	+	-	0	N/D	N/D		+	-	0	N/D	N/D
	Vulnerability	-	0	-	-	-	N/D	N/D		-	-	-	N/D	N/D
	Complexity	-	0	-	-	-	N/D	N/D		-	-	-	N/D	N/D
	Biological risk	-	0	-	- -	- -	N/D	N/D		-	- -	- -	N/D	N/D
	Technological risk: Hazardous substances	-	0	0	0	0	N/D	N/D		0	0	0	N/D	N/D
	Technological risk: Explosions and fires	-	0	0	0	0	N/D	N/D		0	0	0	N/D	N/D
Environment	Global warming	t CO2 eq. / kg 9-cis β-c.	-	-	-	-	+	+		+	0	0	+	++
	Energy resources	GJ / kg 9-cis β-c.	-	-	-	-	+	+		+	0	0	+	++
	Acidification	kg SO2 eq. / kg 9-cis β-c.	-	-	-	-	+	+		+	-	-	++	++
	Eutrophication	kg PO4 eq. / kg 9-cis β-c.	-	-	-	-	+	+		+	0	0	++	++
	Photochemical smog	kg ethene eq. / kg 9-cis β-c.	-	-	-	-	+	+		+	-	0	+	++
	Ozone depletion	g CFC-11 eq. / kg 9-cis β-c.	-	-	-	-	+	+		+	0	0	++	+
	Human toxicity (respiratory inorganics)	kg PM10 eq. / kg 9-cis β-c.	-	-	-	-	+	++		+	0	0	++	++
	Freshwater use (global)	m^3 / kg 9-cis β-c.	- -	0	+	+	+	0		+	+	+	+	+
	Water (local)	-	-	0	0	0	0	0		+	+	+	+	+
	Soil	-	-	0	0	0	0	-		+	+	+	+	+
	Fauna	-	-	0	0	0	0	-		+	+	+	+	+
	Flora	-	-	0	0	0	0	-		+	+	+	+	+
	Landscape	-	-	-	-	-	-	-		0	0	0	0	0
Economy	Operating Expenditure	Million €/year	+	++	+	+	++	++		+	-	-	+	++
	Total Revenue	Million €/year	- -	- -	- -	- -	- -	- -		0	+	+	0	- -
	Gross Margin	%	- -	-	-	-	-	- -		+	+	+	+	-
	Capital Expenditure	Million €	0	0	0	0	++	++		0	0	0	++	++
	Economic Internal Rate of Return (10 years)	%	- -	- -	- -	- -	- -	- -		+	0	0	++	- -
	Net Present Value (10 years, 5% discount)	Million €	- -	-	- -	-	-	-		+	+	+	+	-
Society	Labor rights and decent work	Risk of negative impact/ g 9-cis β-carotene	+	-	- -	- -	+	- -		+	-	-	+	- -
	Health and safety		-	- -	- -	- -	- -	-		0	-	-	0	-
	Human rights		-	0	-	-	++	-		+	-	-	++	0
	Governance		-	-	- -	-	-	+		+	0	+	+	+
	Community infrastructure		+	+	-	-	+	- -		+	-	-	+	- -

BENCHMARK

Fig. 24. Exemplary benchmarking result of the integrated sustainability assessment

The following lessons learned can be extracted from the conclusions and recommendations:

1. *Dunaliella* cultivation and processing requires high expenditures.
   - Processes need to be and can be optimised.
   - Sustainability assessment helps to identify suitable measures.
2. Whether *9-cis* β-carotene provides a new health benefit or not determines the degree of required optimisations. Sustainability impacts seem acceptable for a new pharmaceutical unless avoidable or excessive but for 'only' a natural nutraceutical higher expectations regarding sustainability should be fulfilled.
   - Verify the novel medical value of *9-cis* β-carotene in an adequate clinical trial.
   - Test not only the pure substance but also *9-cis* β-carotene in mixtures.
3. In the analysed scenarios, downstream processing causes by far highest burdens, risks and costs.
   - The modular high-performance countercurrent chromatography (HPCCC) system newly devised within this project should be realised in a relevant environment and evaluated for sustainability impacts.
   - As a fallback option, if *9-cis* β-carotene shows sufficient pharmaceutical efficacy already in extracts, they should not be purified further to avoid environmental burdens and social risks.
4. Site selection is crucial in particular for *Dunaliella* cultivation.
   - Integrate with existing salt activities.
   - Flue gas needs to be, and waste heat should preferably be, available e.g. from a power plant.

- Do not use arable land (exceptions subject to conditions).
- Guarantee sufficient availability of freshwater.

5. Social risks need to be managed
   - High social risks are not a no-go but entail obligations. E.g. closely monitor situation to avoid negative social impacts.
   - Select suppliers according to social reporting standards such as GRI.
6. Solar power can make a big difference.
   - Use as much of own renewable energy, in particular photovoltaics, as possible for algae cultivation.
7. Feed production makes some money and enormously improves land use related environmental burdens.
   - Continue to establish defatted powder as chicken/fish feed.
   - Research feed value of all other lower value biomass streams.
   - Convert all algae constituents to products.
   - Continue to research use of defatted powder in novel foods as substitute of fish-based ingredients.
8. Boundary conditions are important for sustainability.
   - Support approval processes as required because regulatory barriers may prevent realisation by SMEs.
   - In the future, solar power may compete for land and CCU/CCS may compete for remaining CO2 sources. Therefore, a coordination of policies is required.

Harvey, P. (2017): Technological Assessment of *Dunaliella*-based algae biorefinery concepts. In: D-Factory project reports, supported by the EU's FP7 under GA No. 613870, University of Greenwich, Greenwich, UK. *www.d-factoryalgae.eu*.

Keller, H., Gärtner, S., Reinhardt, G., Rettenmaier, N. (2017): Environmental assessment of *Dunaliella*-based algae biorefinery concepts. In: D-Factory project reports, supported by the EU's FP7 under GA No. 613870, IFEU - Institute for Energy and Environmental Research Heidelberg, Heidelberg, Germany. *www.ifeu.de/algae*.

Keller, H., Reinhardt, G., Gärtner, S., Rettenmaier, N., Goacher, P., Mitchell, R., Peñaloza, D., Stahl, S., Harvey, P. (2017): Integrated sustainability assessment of *Dunaliella*-based algae biorefinery concepts. In: D-Factory project reports, supported by the EU's FP7 under GA No. 613870, IFEU - Institute for Energy and Environmental Research Heidelberg, Heidelberg, Germany. *www.ifeu.de/algae*.

Mitchell, R., Goacher, P. (2017): Final report: Economic Assessment of *Dunaliella*-based algae biorefinery concepts. In: D-Factory project reports, supported by the EU's FP7 under GA No. 613870, Hafren Investments Ltd., London, UK. *www.d-factoryalgae.eu*.

Peñaloza, D., Stahl, S. (2017): Final report on social assessment. In: D-Factory project reports, supported by the EU's FP7 under GA No. 613870, Research Institutes of Sweden (RISE), Stockholm, Sweden. www.d-factoryalgae.eu.

11. An Innovation Platform to serve as an interactive forum able to capture the interests and activities of a wide range of stakeholders and integrate their local knowledge and experience with data from the D-Factory partners so that existing knowledge can be shared and new knowledge created.

The D-Factory website has been created to offer support to stakeholders seeking to understand and learn more about the opportunities and benefits of sustainably cultivating and processing halophytic algae for carotenoids, lipids and glycerol, globally. However it also incorporates a link to the D-Factory Platform, which serves as an interactive tool and offers the opportunity to bring together stakeholders focused on establishing algae biorefineries, with D-Factory specialists though its use. The hope is that with better coordination of policy and procurement aligned with adoption of

sustainable technologies exemplified by the D-Factory, a step-change in performance, in the quality of public services and in the ability of businesses will be possible.

The tool is a demonstrator software (Fig. 25 and Fig. 26) and exploits Information and Communication Technology (ICT) Infrastructures. It offers support and online services to all stakeholders with an interest in *Dunaliella* production and processing seeking to learn more about the topic and commercial opportunities. It links produced knowledge and data among the D-Factory partners with a high-throughput screening platform (part of the D-Factory Platform) based on the screening models and allows the user to set certain constraints and manage the results extracted from the model. A comprehensive User Guide is also provided to help users find out how to use the tool and what type of information they may retrieve.

More specifically, it accommodates the following:

- Dynamically builds and visualises the synthesis pathways from algal strains using modelling components.
- Displays the processing paths by utilising the constructed process designs and integrated schemes.
- Screens for optimal paths and highlights solutions.
- Provides alternative solution paths and calculates the corresponding output data.
- Enumerates profit, capital and operating cost and revenue values for each solution path and plots the resulting column chart.
- Outlines the product and technology percentage frequencies through bar charts considering all the selected solution paths.
- Evaluates a set of case studies in order to benchmark a wide range of products and pathways.

In order to reach stakeholders and support a specifically targeted approach a Stakeholder database was created, populated with details of >600 stakeholders and will be used to pro-actively disseminate the availability of the Innovation Platform and knowledge base of the Website..

Fig. 25: The D-Factory Innovation Platform, located at http://snf-777106.vm.okeanos.grnet.gr/dfactory/dplatform/web/index.php?r=site%2Fmodelling

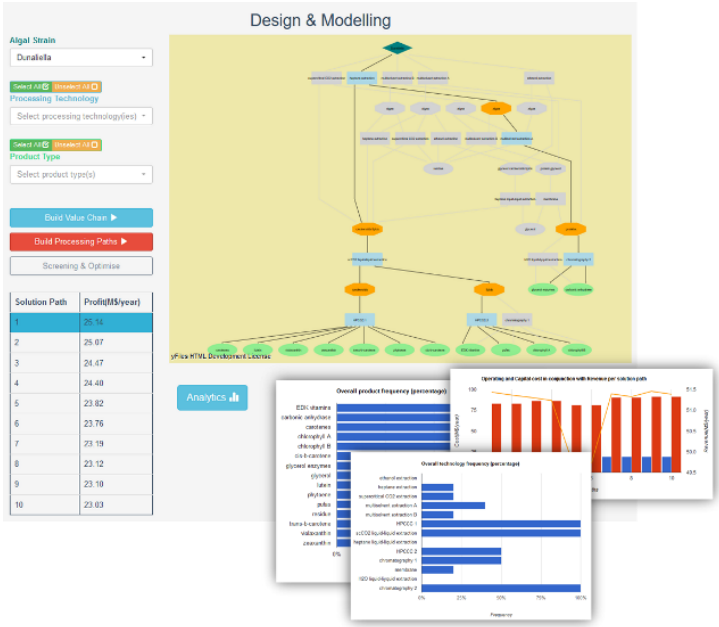


Fig. 26: Value chain optimization and technology integration outputs from use of the D-Factory Innovation Platform

12. A comprehensive business case to show industrial investors the complete opportunity and scope for the creation of a Dunaliella microalgal bio-refinery and raise additional investment for the D-Factory demonstration.

A value chain has been designed to take the D-Factory CO_2 Algal Biorefinery forward into full commercial operation and match production capacity with anticipated demand for carotenoid extract and defatted powder. Dried product from cultivation sites would be collected and shipped by a marketing- management organisation to its warehouses until sufficient material had accumulated for contract processing, initially using supercritical CO_2. The products (defatted biomass and carotenoid extract) would be returned to the company, which could then negotiate prices and ship products to customers. Additional technologies such as HPCCC could be embraced as market demand for carotenoid isomers was proven, and the technologies matured sufficiently for industrial-scale operation.

The Business Plan for global investment in the Algal Biorefinery was developed based on a thorough economic assessment coupled with discussions with several potential customers and collaborators in order to open up possibilities for commercial development. The business plan anticipates four business units each with the potential to attract investment, but over different timeframes. The entire algal bio-refinery would eventually link both upstream cultivation and downstream processing operations, but not all are sufficiently profitable or of sufficient scale to justify external investment. Consequently the recommendation was made that four businesses should be developed separately. **Business One, 'Algal Strains'**, should centre on an operation that uses efficient and proven techniques to identify and develop strains of promising halotolerant microalgae, specifically *Dunaliella salina*. It should capitalise on specialist know-how in strain improvement coupled with their cultivation for specified biomass components and could support other related projects and academic research.

Business Two, "The Farm," should centre on the growth and harvesting of micro-algae, specifically *Dunaliella salina* strains at Monzon, Spain. Monzon data are restricted to the results of a small-scale operation, but were extrapolated to a 10 year investment cycle and the expectation of economies of scale in comparison with the commercial plant of NBT in Eilat, Israel. The Monzon plant was determined to be currently unprofitable but an IRR of 54% is possible with the β-carotene powder priced at €200/kg over a 10 year period. However, an ROI of 11% for the first 5 years moving to 22% over 10 years does not fully justify external investment. Further improvements in production costs or efficiency and a lower price of brine (currently €7.2/m3) or the development of higher added value products would be required to justify a large-scale commercial operation.

Business Three "Supercritical CO_2 extraction" should centre on delivering a high quality 30% β-carotene product as a precursor to pure compounds or a primary high-grade oily extract of carotenoids and a defatted powder using supercritical CO_2 as solvent extractant. Since supercritical CO_2 operations are exceptionally expensive to develop and need to be large scale for cost efficiencies, a third party contract with a preferred large supplier on a sub-contract basis would be required. The pricing of the oil would need to be at least 6.6 X higher than the β-carotene powder to justify the extra investment. Conversely, the cost of extraction should be reduced from the €14/kg to €5-7/kg at volume to fully justify using this process.

Business Four should deliver a series of purified carotenoid isomers. The individual isomers should be used initially for research purposes concerning anti-oxidant benefits. Successful trials would lead to human studies and a premium price of the isomers on success, with a return of 296 %, provided the solvent extraction process using HPCCC separation of carotenes and xanthophylls could be optimised and a commercially viable process found for isolation of the isomers of β-carotene on a consistent basis.

Potential Impact

The D-Factory project has made enormous strides in reducing the technical bottlenecks and increasing the likelihood for investment in a CO_2 microalgal biorefinery, which delivers high added-value products for food, feed, cosmetics and nutraceutical markets, and strengthens the competitiveness of the European marine biotechnology industry.

Dunaliella salina is renowned as the richest source of natural orange, yellow or red pigmented carotenoids. Apart from their colour, these carotenoids may offer protection from compromised immune response, premature ageing, cancers, cardiovascular disease and arthritis. This is important because there are currently no treatments for diseases such as retinitis pigmentosa, atherogenesis and others related to retinoids. So far, research has been limited to the use of food supplements enriched with crude powders of unprocessed *Dunaliella salina*. Establishing exactly which the active agents are, will pave the way for pharmaceutical development to potentially treat **all** diseases where retinoids have been implicated. The active compound has been linked to the presence of *9-cis* β-carotene in the algal powders, but since the isomer cannot be synthesised easily for testing and clinical trials, large amounts of enriched preparations of the isomer have been required. Until now, there has been little justification for investment to produce *9-cis* β-carotene from *D. salina* at the scale required for the trials.

The D-Factory has now developed and detailed a portfolio of products and processes which include, for immediate term development, bioactive extracts enriched in the target *9-cis* β-carotene isomer. From initial tests with this extract, further positive justification for targeting this isomer for clinical trials has emerged. The economic drivers from this development are difficult to estimate but by way of example, if we assume 1 tonne of *Dunaliella* would produce 4,000,000 capsules of stereoisomeric active *9-cis* β-carotene ingredient and if we assume a 5% population demand then this would represent a daily dose for 200,000 people per year.

The D-Factory portfolio also includes natural colorants as a by-product of processing for *9-cis* β-carotene, which are well-suited for food markets that are growing to meet demand from the LOHAS (Lifestyles of Health and Sustainability) demographic or other food sectors. The food colorant market is one of the major segments of the global food additives market. Food colours are used by the food and beverages industry, to improve and impart colour for the visual appeal of the food which is lost while food processing.

After extraction of enriched lipophilic carotenoids, a defatted powder additive for high-value feed is obtained. This contains carbohydrates and proteins able to deliver substantial reductions in environmental burdens by substituting conventional feeds. However, chick-feed trials also indicate it contains an active ingredient that delivers a good feeding response at low dose. This suggests a product which might be used to help in the drive to find alternatives to growth promoting antibiotics (banned in the EU since 2006) in animal feeds and additional trials are now planned.

The algal strains required for producing the D-Factory portfolio of products are halotolerant, hyper-accumulating carotenogenic strains of *Dunaliella salina*. These are now cryopreserved, patented and characterised as D-Factory (DF) strains, which are available to global communities by application to the MBA Culture Collection https://www.mba.ac.uk/facilities/culture-collection#b17, where a video entitled “Algae strains in D-Factory” is also available. Strains DF15 and DF40 are also deposited with an International Depository Authority Application, and will also maintain traceability according to the Nagoya Protocol. Characterisation of the properties of the strains, described in Patent application numbers 1701855.7 and 1702413.4 (Algal Composition) underpins the know-how of the D-Factory partners to identify, isolate and develop strains of promising halotolerant microalgae, globally. The skills are available to support stakeholders seeking to establish a business in Algal Strains for specified biomass components, as detailed in the D-Factory

website (Outcome Statements). The offering has also been disseminated to research and other interested communities through oral presentations at specialist conferences e.g. International Society of Applied Phycology, 2017; BBSRC NZ-UK Algae Research Meeting; and at the European conference, Algae Biorefineries for Europe, 2017 https://algaebiorefineryconference.eu/wp-content/uploads/2017/11/S16.pdf. The characteristics of the strains have been highlighted by comparing these with other strains in a special issue publication of Microalgal Biotechnology Biology (2018).

Success in planned trials of animal feed and carotenoids extracts with external stakeholders could lead to substantial customer demand for *Dunaliella* algal biomass processed products: at an inclusion rate of 100g algae defatted powder/tonne approximately 400 tonnes defatted biomass would need to be targeted initially, and would simultaneously provide at least 70 tonnes pa carotenoid extract for food colouring markets.

This demand could be met, sustainably, by adopting D-Factory cultivation know-how that has now been developed to cultivate the D-Factory algal strains throughout the year. The know-how includes capturing flue gas CO_2 from the combustion of natural gas for heat and power; controlling seasonal temperature variations with heat/cooling systems in more northern hemispheres and integrating production with a salt production facility. It also includes how to double algal biomass yield, and control predators; how to integrate *Dunaliella* cultivation in PBRs for inoculation purposes; how to culture in brine with the correct composition and how to stimulate production of the colourless carotenoids phytoene and phytofluene using new generation herbicides. Furthermore, a novel cultivation method for producing *Dunaliella* algal powders with a more than 2-fold increase in *9-cis* β-carotene is now captured in Patent application number 1718822.8 (Method of treatment, production of powder and processing for β-carotene) details of which will be published and shared with the research community to advance our understanding of the biochemical processes, once successfully patented.

An Integrated Assessment of Sustainability published by IFEU and available on their website and that of the D-Factory has recommended integrating cultivation with existing salt activities; using flue gas and waste heat from a power plant; avoiding using arable land and guaranteeing sufficient availability of freshwater. This know-how is demonstrated at the Monzon pilot facility in Spain, where Monzon Biotech is poised to match the anticipated demand for dried algal biomass by organising production of the required microalgal quality with as many additional farms as required and driving forward further improvements in production costs or efficiency with an application for SME-Instrument funds and a lower price of brine. Additional algal cultivation sites could be remote from Monzon, but expansion locally might also be possible with additional land purchase. The farms, if several were involved, might be operated as a franchise.

Know-how to double biomass yield is being exploited by NBT to increase its production of *Dunaliella* powder to meet the growing demand from markets in China and Japan. There is also interest for a huge project for mass cultivation / harvesting of *D. salina* in East India. A commercial case for cultivating and harvesting *Dunaliella* at scale is now available for investment considerations to facilitate the step-change needed in production capacity in Europe. Highlights of the cultivation know-how and opportunities for large-scale expansion have been disseminated at conferences such as Algal Biorefineries for Europe, 2017 and highlights are available from presentations available at https://algaebiorefineryconference.eu/wp-content/uploads/2017/11/S05_Algae-Biorefineries-for-Europe-D-Factory-Ami-Ben-Amotz-2017.pdf (Cultivation of *Dunaliella* in large-scale open raceways for the commercial production) and at https://algaebiorefineryconference.eu/wp-content/uploads/2017/11/D-FACTORY-AM-BEN-AMOTZ.pdf (Large scale open ponds commercial cultivation of *Dunaliella* as part of the D-Factory project).

Current practice for harvesting *Dunaliella salina* from saline culture medium centres on the use of well-established disc-stack centrifuges, but the cell wall-less cells are very fragile and easily ruptured in the course of harvesting, which increases the organic matter content of harvesting effluent and increases costs for effluent treatment before discharge to water-courses. To drive biomass production costs down, D-Factory partners have been developing a process with Evodos spiral plate technology to harvest the fragile halotolerant algae intact. This process could reduce waste and reduce costs in water treatment.

The Evodos 50 A prototype was developed and tested in collaboration with NBT in the D-Factory project and now harvests *D. salina* cells continuously at 2,500 $L.h^{-1}$, with 95% separation efficiency and ~90% intact, to provide a paste of 40% solids; the smaller batch harvester Evodos T10 harvests at 350 $L.h^{-1}$, with 95% separation efficiency and cells >95% intact, to provide paste of 20-30% solids. These Evodos technologies (TRL5) still require some further technical development to reduce costs and further cycles of harvesting are needed to demonstrate cell survival and to provide values of organic matter reduction in effluent/recycle stream, but once developed to maturity, they are likely to enormously reduce costs of effluent discharge from harvesting *D. salina*.

There are early indications that significant progress to reduce costs can be made by incorporating pre-concentration membranes in-line with Evodos technologies and using advanced oxidation techniques (UV with H_2O_2) to treat the effluent. The approach was developed in the D-Factory project using a technique for monitoring cell integrity based on fluorescence and is now widely available through a publication with open access in Algal Research 24 (2017) 325–332. Harvesting of *Dunaliella salina* by membrane filtration at pilot scale has also been published in J. Separation and Purification Technology 190 (2018) 252-260 seppur.2017.08.019 and presented as both oral and poster presentations at conferences e.g. the International Congress on Membranes and Membrane Processes; and Algal Biorefineries for Europe https://algaebiorefineryconference.eu/wp-content/uploads/2017/11/S24.pdf; Harvesting of fragile algae *Dunaliella salina* by membrane filtration with permeate recovery for microalgae cultivation, at https://algaebiorefineryconference.eu/wp-content/uploads/2017/11/D-Factory-EVODOS.pdf

Once scale-up in production has been achieved, industrial-scale processing of algal biomass is required in order to prepare an extract enriched in *9-cis* β-carotene and the co-product, defatted powder. Using supercritical CO_2 ($scCO_2$), the D-Factory project has developed a process to prepare a high quality 30% β-carotene product as a precursor to pure compounds or a primary high-grade oily extract of carotenoids, and defatted powder. Both fractions are free from petrochemical solvent. The know-how is held in-house by NATECO and is now available in process contracts to supply carotenoid extracts and defatted powders from algal biomass powders. However, for processing using $scCO_2$, the algal powders need to be of a certain critical particle size. Freeze-dried powders meet the specification needed, but the process is more expensive than spray-dry technology. Further development of spray-dry technology to deliver the required particle size specifications would further reduce costs in algae powder production.

Further processing of carotenoid extracts to deliver single carotene isomers is extremely challenging in scale-up. However preparations of extracts highly enriched with *9-cis* β-carotene can be prepared quite simply now, by processing algal powders specifically tailored for this purpose and detailed in Patent application number 1718822.8 (Method of treatment, production of powder and processing for β-carotene). Tests of these with flies and mice have provided extremely promising data and will be published with Moorfields Eye Hospital & UCL Institute of Ophthalmology and made available on the D-Factory website to encourage investment in clinical trials of *9-cis* β-carotene-enriched extracts prepared by “Algae Farms”.

Preparations of extracts highly enriched with *9-cis* β-carotene (> 90% w/w *9-cis* β-carotene, ~90% total carotenoids) and stable, can also be prepared from solvent extracts of algal biomass powders

using an anti-solvent, or by freezing: this step separates *all-trans* β-carotene from enriched preparations of carotenoids and can be used as a food colorant in a solvent processing step, as detailed in https://algaebiorefineryconference.eu/wp-content/uploads/2017/11/D-FACTORY_C-Wosu.pdf and https://algaebiorefineryconference.eu/wp-content/uploads/2017/11/D-Factory_David-Rooke.pdf and available on the D-Factory website. The information will also be published as part of a PhD Thesis in 2018 and in manuscripts prepared for the research community. The full integrated process still requires development, especially to remove solvents from products and allow solvent re-cycling and recovery in processing. The use of membranes has been tested in the D-Factory project and they show promise (TRL2) but complete membrane selection/development work will be required for all solvents selected for an integrated process.

Stabilising carotenoid extracts and formulating them for use by customers is crucial for developing an enduring D-Factory value chain that brings new products into the market. To that end D-Factory know-how has been developed to add further value to carotenoid extracts in several ways: Oil-dispersible powders of carotenoid-rich extracts by PGSS technologies are now available. PGSS technologies produce different product forms with easier handling and a longer shelf life. The powders are also easier to standardize with respect to carotenoid content.

A water-dispersible mesoporous silica delivery system has been developed for carotenoid-rich extracts. Loading of carotenoids (and poorly soluble materials in general) into porous particles enables improved bioavailability upon e.g. oral administration. Silica particles containing up to 4% of extract and with excellent powder flow and dissolution properties have been produced, ready for further development for the upscaling to industrial scale and for optimizing loading, stability and release.

The technology for the production of water-dispersible microencapsulated powders by spray-drying has been developed. Oil-in-water nanoemulsions and solid lipid nano-carriers food and nutraceutical delivery systems for carotenoid extracts have been prepared and characterised. The details are available in two MSc theses which are publically available: 1) Emulsification strategies for the preparation of nanoemulsions containing lutein; 2) Formulation of Delivery Systems for Carotenoid-Rich Extracts from Microalgae. These need to be optimised for chemical stability and developed for the upscaling to industrial scale. A pharma grade formulation of carotenoid-rich oils into oil-in-water nano-emulsions has also been developed in the D-Factory project, and these are now distributed for testing by a stakeholder.

For all these developments prototype applications are available to all interested stakeholders through the D-Factory application notes (https://www.d-factoryalgae.eu/profiles/dfactory/images/file/D-Factory_Application-Note.pdf), and many have been described in the D-Factory- branded outcomes statements and in presentations given at the Algal Biorefineries for Europe, 2017 conference https://algaebiorefineryconference.eu/documents/ to encourage further development by stakeholders.

The defatted powder prepared from processing with $scCO_2$ is rich in essential amino acids and omega 3 fatty acids and its development as a chick feed additive has been the subject of a PhD project. Success in further feed trials will prove the value of the defatted biomass as chick feed for large-scale commercialisation. Initial results of the use of the material as a possible antimicrobial additive have been published to enable rapid progression towards end-user take-up of materials and details have been freely disseminated to all interested stakeholders through the D-Factory branded outcomes statements and conference poster Potential of supercritical CO_2-extracted, defatted *Dunaliella salina* biomass as a broiler feed ingredient, https://algaebiorefineryconference.eu/wp-content/uploads/2017/11/D-Factory_P-J-Sanderson.pdf. The aim here is to encourage investment in “Algae Farms” outlined above.

D-Factory partners have also demonstrated that it is possible to make appetizing-looking, gluten-free bread, with good structure using the *Dunaliella* Albumin protein fraction. Development is needed for the upscaling to industrial scale and for optimizing formulations such as bread recipes and meeting EU Novel Foods legislation. A process using $scCO_2$ to prepare defatted biomass for novel foods such as fish sausages has also been developed by the D-Factory project. As with the bread, further development is needed for the upscaling to industrial scale, for optimizing formulations and for meeting EU Novel Foods legislation. It is also possible to isolate *D. salina* starch and its properties. A process for preparing polar lipids from *Dunaliella* biomass and using them in formulations has also been developed in the D-Factory project. The characterisation of the emulsification properties of polar lipid fractions isolated from *Dunaliella salina* showed that the hydrophilic-lipophilic balance (HLB) ranges from 14 to 5. These polar lipid fractions are suitable for stabilisation of emulsions of vegetable, mineral and silicone oils.

The information and prototype applications for each of these products, gluten-free bread, fish sausage, starch and lipid emulsifiers, has been freely disseminated to all interested stakeholders through the D-Factory application note (https://www.d-factoryalgae.eu/profiles/dfactory/images/file/D-Factory_Application-Note.pdf), and in the D-Factory branded outcomes statements and conference presentations to encourage further development by stakeholders.

High Performance Counter-Current Chromatography (HPCCC) technology has also been developed in the D-Factory project, described in Patent Application (Patent application reference number 1718939.0; filing date 16 Nov 2017; new modular design processor system covering modular designs for all core processors, all column volumes and all CCC/CCS processing systems). Development and production of the new modular HPCCC systems to run continuously 24/7 takes the DE technology to a completely different level as an industrial processing tool: production of the first is planned for Q2/3 this year. Its use to prepare extracts enriched in lutein and zeaxanthin (>90% pure lutein and zeaxanthin (and other xanthophylls)) has been detailed to reach all interested stakeholders through the D-Factory brochure and in the D-Factory branded outcomes statements, conference presentations and application notes (https://www.d-factoryalgae.eu/profiles/dfactory/images/file/brochure_dfactory.pdf and https://www.d-factoryalgae.eu/profiles/dfactory/images/file/D-Factory_Application-Note.pdf), to encourage further development by stakeholders. The technology (TRL2/TRL3) requires optimisation of the conditions to separate xanthophylls from chlorophyll / pheophorbide and to provide demonstrable evidence of purity. Once developed, the technologies will be available to add further value to carotenoid extracts in the form of pure analytical reference standards especially *9'cis* β-carotene; lutein, zeaxanthin and potential other high purity target compounds and to prepare and apply for a monograph to pharmacopoeia commission(s).

Dunaliella-specific, D-Factory technologies that have been developed in the course of the project are also having an impact on the SME businesses of partners in the project.

The know-how introduced by A4F on the design and adaptation of PBR systems for *Dunaliella* cultivation is being actively exploited for microalgae production unit deployment by A4F, in both stand-alone and in integrated co-location with industry. A4F has also developed new know-how to cultivate carotenogenic orange, green phase and brown phase *Dunaliella* in green walls; tubular PBRs and pilot scale cascade raceways and is poised to produce small batches of *Dunaliella* biomass with required properties for research purposes. For the technologies requiring further development to achieve commercial exploitation status, A4F will apply for further research grants to help fund the development, and also mobilize internal efforts for that purpose. These D-Factory technologies are expected to make a significant contribution for the A4F track record presented to clients interested in implementing microalgae production facilities. Some examples are the

deployment of PBR systems for the fragile and challenging microalgae *Dunaliella*, the utilization of cascade raceways for *Dunaliella*, the ability to cultivate *Dunaliella* in different growth stages, including highly carotenogenic, in outdoor conditions, and the ability to integrate production with harvesting intact cells with Evodos dynamic settlers, using membrane pre-concentration and reuse culture medium. The outcome of the D-Factory is, thus, in line with A4F's business of providing clients with microalgae production facilities and consultancy on microalgae cultivation. A4F forecasts for the next 2-3 years to deploy, for its clients, between 1 and 3 industrial scale microalgal production facilities growing *Dunaliella*, and to supply services of small scale biomass sample production and consultancy for 8-12 clients in the same timescale. Half of the business opportunities are expected to come from outside of Europe.

Evodos also sees a huge potential for harvesting algae using the technologies they were able to develop further in the D-Factory. After the Evodos T50 was launched at NBT, Evodos developed the next generation Evodos 50 and this is now installed to harvest a range of microalgae 24/7 without any issues. They are also developing new Cleaning in Place (CIP) procedures, which were highlighted in the D-Factory as needing attention and this is expected to be finished by Q3 2018. They have also just finished commissioning an Evodos algae harvester in India where there is a huge algae pilot facility and once completed the intention is to scale up to massive production.

The project has also contributed to the establishment of a *D. salina* research and technology community with a high level of know-how and engagement, made up of scientists who have been willing to partner and combine their efforts to tackle the major challenge now facing commercialisation of algal based products, namely the European regulations. Clear directives and support is needed to facilitate commercialisation of algal-based products and extracts in pharma, food, cosmetic and nutraceutical applications. With these in place, B2C and B2B industries driven by consumer/regulatory demands on natural and bioderived products will be able to commit and provide the pull and support needed to tackle the economic viability challenges. The research community also benefits from shared knowledge developed by the D-Factory scientists in analysis, for example, in separating and quantifying carotenoids.

Improved understanding by stakeholders of the opportunities inherent in the D-Factory concept is also facilitating development of a new national project in Greece built along D-Factory lines. This was helped by use of the D-Factory Platform Tool designed to encourage both uptake and training of the concepts behind the D-Factory algae biorefinery and available on the D-Factory website at https://www.d-factoryalgae.eu/index.php?id=4&lang_id=eng. The intention is to explore D-Factory products as ingredients for the food industry. Superfoods®, a major producer of a nutraceuticals and pharmaceuticals conglomerate in Greece is undertaking the formulation of a wide range of end-user products that will be based on D-Factory chemicals and the National Technical University of Athens will explore the production of *Dunaliella salina* setting up a pilot facility in Lavrion, a small port South of Athens. The project is supported by the General Secretary of Research and is expected to start in the summer of 2018.

The portfolio of consistently assessed examples of algae cultivation and use that has now been developed by IFEU is unique because it ranges from extension and improvement of existing industrial-scale facilities operational for decades, to new concepts. The technologies developed for algae cultivation and use in the D-Factory project were analysed using an exceptionally comprehensive range of methodologies covering all relevant environmental, economic, social, technological and regulatory aspects. The insights gained have been condensed into lessons learned with concrete recommendations on how to improve the sustainability of algae cultivation and use in general and nutraceutical production in particular. These have been widely disseminated to all interested stakeholders via many European and international conference presentations and

the D-Factory-branded outcomes statements to encourage future developments for sustainable production in the algae industry in general.

Many of the D-Factory technologies and supply chain know-how and understanding was presented at the two-day international conference held in October 2017, Algae Biorefineries for Europe https://algaebiorefineryconference.eu/, which was jointly organised to showcase the four European collaborative R&D projects – Bisigodos, D-Factory, Miracles and PUFAChain to more than 100 stakeholders from industry, governance, policy makers, public authorities, academia and finance and inform them of the potential of algae biotechnology for sustainable development. Presented together, the conference provided an important opportunity to position the D-Factory as a whole, globally, including to members of the research community from China and the US, to increase the potential impact of European-sponsored biotechnology.

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