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Towards a sustainable *Dunaliella salina* microalgal biorefinery for 9-*cis* β-carotene production

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ABSTRACT

Valorisation of the efficacy of *9-cis* beta-carotene in treating atherosclerosis, psoriasis, and inhibiting atherogenesis and retinitis pigmentosa is becoming increasingly urgent, but supplies of *9-cis* beta-carotene are scarce and this compound is difficult to synthesise chemically, unlike the much more common *all-trans* form. Innovative products, processes and services in an algal biorefinery that rely on renewable biological resources instead of fossil fuel alternatives offer the potential to lower the energy costs of traditional chemical processes and reduce carbon emissions, water usage and waste. In 2013, the European Commission supported development of 4 microalgal biorefinery projects to assess the potential for innovative approaches to tackle the major challenges intrinsic to the development of the algae biorefineries. One of these was the D-Factory (KBBE.2013.3.2-02) which sought to evaluate requirements for sustainable, industrial-scale production of *Dunaliella salina* and extraction of its carotenoids, especially *9-cis* beta-carotene in a CO_2 microalgae biorefinery. Here we present findings of the D-Factory project and propose a way forward for industrial-scale production of *9-cis* beta-carotene using biotechnology based on *Dunaliella salina* biomass. Cultivation improvements are able to deliver more than double the current levels of productivity, with increased sustainability, whilst the use of natural hyper-accumulating carotenogenic strains combined with the use of red light to boost production of the beta-carotene pathway, will increase the relative concentration of *9-cis* beta-carotene in extracts of carotenoids with consequent improvements in downstream processing. These developments pave the way for acquiring data for a Medicine Licence and prepare the market for entry of novel *9-cis* beta-carotene products.

1. Background

In the field of biotechnology, the use of microalgal biomass resources is evolving exponentially through research efforts to exploit algal metabolism to synthesise and accumulate a wide range of compounds of industrial interest, as well as lower the energy costs of traditional chemical processes and reduce carbon emissions, water usage and waste [1]. However, to realise the 'green gold' potential of microalgae successfully and establish the validity of green approaches over fossil-based systems, requires strategic planning. In 2013, the executive branch of the European Union, the European Commission, supported development of 4 microalgal biorefinery projects to assess the potential for innovative approaches to tackle the major challenges intrinsic to the development of the algae biorefineries for production of high value-added products such as polymers, pharmaceuticals, oils and chemicals, bioactive compounds and colorants. A further aim was to deliver a robust scientific and technological basis for substantiating strategic decisions for the industrial development of algae for high added-value products.

One of these was the D-Factory (KBBE.2013.3.2-02) [2], which sought to evaluate requirements for sustainable, industrial-scale production of *Dunaliella salina* and extraction of its carotenoids, especially *9-cis* β-carotene, in a CO_2 microalgae biorefinery.

1.1. The carotenoid 9-cis β-carotene

Carotenoids are conjugated isoprenoids which are synthesized by all photosynthetic organisms for light-harvesting and for photo-protection. Carotenoids that contain at least one unsubstituted β ring are critical for the vitamin A pathway and needed in the human diet to produce retinoids [3]. These play important roles in cell differentiation, growth, and apoptosis (for reviews see [4–7]). They are also critical for controlling vision defects [8]. Intact carotenoid molecules and carotenoid cleavage products other than retinoids may also act as lipid radical

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scavengers and as singlet oxygen quenchers to protect against oxidant stress [9], as well as act as blue light filters for photoprotection of eyes and skin [10]. They are also attracting interest as promoters of cognitive functions [11]. The carotenoids market is projected to grow to USD 2.0 billion by 2026, largely due to increasing use of natural carotenoids especially β-carotene as food colorants, and to innovations in extraction technologies [12].

9-cis β-carotene is one of the most potent precursors of retinoids. Recent evidence has suggested that *9-cis* β-carotene might serve as an effective treatment for a range of retinoid dystrophies, including type 2 diabetes, obesity, certain types of cancer (breast, cervical, ovarian, colorectal), mild, chronic plaque psoriasis and a range of cardiovascular diseases [13–24]. With an aging society across Europe and much of the world, the development of an effective product to treat life-changing conditions and combat common eye disease and blindness would bring significant societal benefits. Atherosclerosis is a chronic inflammatory disease of the arteries and underlying cause of ~50% of all deaths in westernized society. An estimated 17.9 million people died from Cardio Vascular Diseases in 2016, representing 31% of all global deaths. Of these deaths, 85% were due to heart attack and stroke (WHO). Retinal degeneration affects millions of people in Europe and world-wide, leading to substantial social and economic burden. Based on [25] we estimate that more than 288 million people around the world will suffer from vision loss due to an inherited retinitis pigmentosa and age-related macular degeneration.

9-cis β-carotene, however, is scarce and difficult to synthesise chemically, unlike the much more common *all-trans* form. The current state-of-the-art production of *9-cis* β-carotene is via an 8-step chemical synthesis based on β-cyclocitral and *all-trans* retinal afforded by the Wittig reaction of all-trans retinal and 9-cis-retinyl triphenylphosphonium bromide salt [24]. The relative difficulty in synthesis of *9-cis* β-carotene is reflected by a price comparison of the two products currently marketed by Sigma-Aldrich (Merck), namely, €6/g for the *all-trans* form, compared to €500,000/g for *9-cis* β-carotene.

Cultivation of[1] *D. salina* could offer a solution, because it produces β-carotene in high concentration of *cis/trans* (Z/E) configurations, ~1:1 (see Table 1) and will accumulate up to 10% of the dry biomass as β-carotene [26–29]. Furthermore, it grows in extreme, salt-water conditions around the globe, at extreme temperatures from around −5° to above 40 °C and at salt concentrations up to NaCl saturation, which cannot be used for agriculture for food production [26]. In these relatively simple hypersaline ecosystems, competition for resources is reduced compared to freshwater systems and the risk of culture crash can be rapidly controlled with NaCl addition: *D. salina* grows optimally at 10–15% salt but will also grow at salt concentrations up to saturation and will dominate ecosystems at the highest salinities where it lacks competitors. Increased salinity up to 25% are typically applied in cultivation to control predators and to optimise carotenogenesis. Consequently, *Dunaliella* can be cultivated in open pond raceways (OPRs), which have lower construction costs and are more easily maintained compared to closed vessel photobioreactors (PBRs) [30]. Cultivation of *D. salina* for *9-cis* β-carotene could therefore reduce the capital expenditure (CAPEX) of *9-cis* β-carotene production several 10's -fold compared to that required for chemical synthesis and represent a significantly more environmentally-sustainable solution for *9-cis* β-carotene production. Cultivation would also lend itself to scale-up production using resource-efficient, low-cost raceways with commensurate increase in CO_2 fixation by photosynthesis.

Nature Beta Technologies (NBT) (Ltd) (Israel) currently produces ~25 t Ash Free Dry Weight (AFDW) *D. salina var bardawil* biomass per annum from 10 Ha of raceways and represents one of the largest reliable sources of *D. salina* powder in the world [2]. However, the powder is not cultivated for high *9-cis* β-carotene content. Monzon Biotech S.R.L. (MB) in Spain produces ~400 kg p.a (AFDW) *D. salina* for established markets, but current biomass productivity ranges of *D. salina* lie between 0.75 g m^{-2} d^{-1} and 3.0 g m^{-2} d^{-1} (AFDW) depending on seasonality and other factors [2]. Other sources of *Dunaliella* carotenoid-containing products exist e.g. Betatene produced by BASF, which is extracted using only natural plant oil, and Solgar Natural Source Oceanic Beta Carotene Softgels, but these are of variable quality (*9-cis* β-carotene/*all-trans* βC-carotene = 0.4 or less).

Table 1
β-Carotene content and isomeric composition.

Source	β-Carotene content % AFDW	β-Carotene composition (%)	
		All trans β-carotene	*9-cis* β-carotene
Synthetic	100	> 98	< 2
Carrot	0.01–0.06	50	2
Palm oil	0.06–0.07	36	24
Dunaliella	6–14	50	> 40

1.2. Biorefinery challenges for producing 9-cis β-carotene by D. salina

Three challenges/questions for producing *9-cis* β-carotene in a biorefinery were envisaged at the start of the D-Factory project:

1.2.1. Question 1: Can the biomass yield of D. salina be increased sustainably?

Reproducible, low-cost production of *D. salina* biomass with a consistently high relative content of *9-cis* β-carotene would be required to convince the manufacturing sector of the commercial viability of adopting new processing practices and engaging with new value chains, and to persuade investors of the viability of investing in new 'farming' practice. It would also be required to support acquisition of a Medicine Licence to underpin nutraceutical claims and drive market demand for products. Furthermore, whilst the expected biomass productivity of microalgae, as a whole, has been estimated at ~25 g $m^{-2}d^{-1}$ based on an estimated 3% photosynthetic efficiency, solar intensity of 4000 kcal $m^{-2}d^{-1}$ and algal calorific value (5 kcal g^{-1}) [31], this is approximately 8-fold greater than achieved by most commercial operators.

Dunaliella carotenogenesis is a stress condition dependent on the light/cell ratio and on the nitrate/cell ratio and salinity [32–35]. Consequently, production tends to vary as a function of season, the availability and nature of nutrient nitrogen supplied, and the level of CO_2, which poses problems for commercial production. With increasing temperature, CO_2 solubility in water is reduced and, at the high temperature and pH usually found in the natural brines in which *Dunaliella* grows, the bulk of the inorganic carbon (> 99%) is in the form of $HCO_2/CO_3{}^{-2}$ and is less available for uptake. Below 30 °C, CO_2 consumption of 10 g m^{-2} d^{-1} is typical for an alga production of 2 g $m^{-2}d^{-1}$, giving a CO_2 Mass Transfer = 5 g/g, but at temperatures > 30 °C the CO_2 Mass Transfer can reach > 10 g/g. Also, above temperatures of 35 °C, glycerol tends to leak from cells, which will support the growth of halophilic heterotrophs including fungi, yeasts and halophilic Archaea (family Halobacteriaceae). Carotenogenic cultures are also much more sensitive to the chemical and mineral composition of the brine and turbulence compared to non-carotenogenic cultures.

Since *Dunaliella* grows well below 16 °C, and the Q_{10} of photosynthesis is quite stable at low temperatures, growth rate can improve with temperature control below 30 °C. Also, lower cultivation temperatures favour increased β-carotene content and higher relative ratio of *9-cis* β-carotene with no significant changes in the other cell pigments [36].

[1] *Dunaliella salina* throughout this manuscript represents also its variant *Dunaliella bardawil*.

Table 2
Typical composition for Dunaliella from the D-Factory, Ash-free dry weight basis (AFDW) [2].

Total protein g kg^{-1} AFDW	Total Lipid g kg^{-1} AFDW	Total carotenoids g kg^{-1} AFDW	Carbohydrate g kg^{-1} AFDW	Glycerol g kg^{-1} AFDW
200–300	100–200	60–90	500–800	> 100

Table 3
Amino acid profile of *D. salina*.

Category	Amino acid	% of total amino acids
Essential amino acids	Histidine	2.6
	Isoleucine	5.7
	Leucine	7.3
	Valine	6.3
	Lysine	2.1
	Phenylalanine	11.5
	Methionine	2.1
	Threonine	7.3
	Tryptophan	2.6
Conditionally essential amino acids	*Arginine	2.6
	Tyrosine	1.0
	Cystine	2.1
	Glutamic	10.4
	Glycine	9.4
	Proline	1.0
Non-essential amino acids	Alanine	7.3
	Aspartic	18.2
	Serine	0.5

* Also essential in the diet of animals.

1.2.2. Question 2: Can 9-cis β-carotene be separated cleanly and in high yield from other components of harvested D. salina biomass?

Carotenogenic *D. salina* biomass after harvest contains a range of polymers in addition to glycerol and carotenoids, which need to be separated from *9-cis* β-carotene (See Table 2).

Secondly, the conjugated polyene chain that is characteristic of carotenoids makes carotenoids susceptible to oxidative degradation [37]. Polyunsaturated lipids including omega-3 essential fatty acids, are also highly oxidizable. Currently the most commonly used extraction methods include organic solvent extraction, which is often assisted by heat, pressure, microwave or ultrasound for cell disruption; and sub- and supercritical fluid extraction [38–42].

The use of supercritical CO_2 ($scCO_2$) to extract carotenoids from biomass minimises exposure to oxygen, is inert, readily available in pure form, and easily removed from products, and selective extractions are possible because the solubility of many extracted compounds in CO_2 varies with temperature and pressure. Use of $scCO_2$ also overcomes limitations to the solubility and polarity spectrum imposed through using the diminishing range of acceptable organic solvents [40,41]. Spent biomass is also stable, because it is defatted, reducing the risk of rancidity and enriched in the more polar polyphenols and proteins. However, the use of $scCO_2$ is not suitable for cells with a high-water content [42], consequently biomass would need to be dried, which is energy-intensive.

9-cis β-carotene also shares very similar chemical properties with *all-trans* β-carotene, the other major carotene present in carotenogenic biomass. Few reports exist on industrial-scale separation of *9-cis* β-carotene from other carotenoids. Haigh (2000) [43] patented separation of a carotenoid extract on a deactivated alumina chromatographic column followed by crystallisation of *all-trans*-β carotene to obtain preparations comprising 50–75% *9-cis* β-carotene and up to 40% all-trans-β carotene and Sibeyn & De Pater, 2002 [44] patented the temperature-induced crystallisation of *all-trans*-β-carotene at low temperature to obtain β-carotene crystals ≥90% purity from *Blakeslea trispora*.

1.2.3. Question 3: Can 9-cis β-carotene be produced sustainably in a biorefinery with lower energy costs compared to traditional chemical processes, and reduced carbon emissions, water usage and waste?

Apart from *9-cis* β-carotene, the carotenoid complement of *D. salina* may include the colorless carotenoids phytoene and phytofluene, lutein and zeaxanthin, and α-carotene, depending on culture conditions.

Phytoene and phytofluene are rarities among naturally synthesized carotenoids [10] but ideally suited to protect the skin against long wave ultraviolet A (UVA) and short wave ultraviolet B (UVB) and offer antioxidant and anti-inflammatory effects [45,46]. Currently more than 90% of the active ingredients in cosmetics are still of synthetic origin.

The human macula uniquely concentrates three carotenoids: lutein, zeaxanthin, and meso-zeaxanthin. Lutein and zeaxanthin must be obtained from dietary sources whilst meso-zeaxanthin is believed to be formed at the macula by metabolic transformations of ingested carotenoids. Clinical trials using lutein and zeaxanthin supplements results in augmentation of macular pigment and consequential benefits in visual performance whilst consumption of lutein- and zeaxanthin-rich products is associated with lower incidence of cancer, cardiovascular disease, Age-related Macular Degeneration and cataract formation. Lutein is clinically proven to prevent cataract and macular degeneration and may function as an anti-oxidant to decrease around 60 chronic disease risks [47].

α-Carotene has proven anti-metastatic action, which is not associated with provitamin A activity, which may involve the mediation of gene expression and signalling pathway related to invasion and migration [48].

The biomass components once separated from carotenoids also present several opportunities for use as by-products. For example, although use of $scCO_2$ uses high temperature and pressure which denatures proteinaceous enzymes, the proteins once separated contain the ten essential amino acids required by animals and humans in their diet [2] (see Table 3). The lipid profile of the powder also indicates value for food, or feed with a high relative content of omega-3 fatty acids [2] (Table 4). Additionally, in a 1000-chick feed study, a low additive inclusion level of *D. salina* defatted powder (0.1–1% w/w) prepared after $scCO_2$ extraction of *D. salina* biomass displayed bioactive properties

Table 4
Fatty acid profile of *D. salina*.

Identified fatty acids	% of TFA	Identified fatty acids	% of TFA
C08:0 caprylic acid	0.00	C18:1 Oleic Acid	5.81
C10:0 capric acid	0.00	C18:2 Linoleic Acid	4.91
C11:0 undecylic acid	5.62	C18:3 Linolenic Acid	17.65
C12:0 lauric acid	0.15	C18:4 Stearidonic Acid	0.26
C13:0 tridecylic acid	0.67	C20:0 Arachidic Acid	0.46
C14:0 myristic acid	1.77	C20:1 Gadoleic Acid	1.18
C14:1 myristoleic acid	0.32	C20:4 Arachidonic Acid	0.00
C15:0 pentadecanoic acid	0.12	C22:0 Behenic Acid	0.31
C15:1 pentadecenoic acid	0.10	C20:5 Eicosapentaenoic Acid	0.00
C16:0 palmitic acid	33.69	C22:1 Erucic Acid	0.00
C16:1 palmitoleic acid	0.72	C22:4 Adrenic Acid	0.00
C17:0 heptadecanoic acid	0.30	C24:0 Lignoceric Acid	0.00
C17:1 heptadecenoic acid	3.21	C22:5 Docosapentaenoic acid	0.00
C18:0 stearic acid	1.77	C22:6 Docosahexaenoic Acid	0.00
Unidentified fatty acids			20.98
Total saturated fatty acids			44.86
Total monounsaturated fatty acids			11.34
Total polyunsaturated fatty acids			22.82

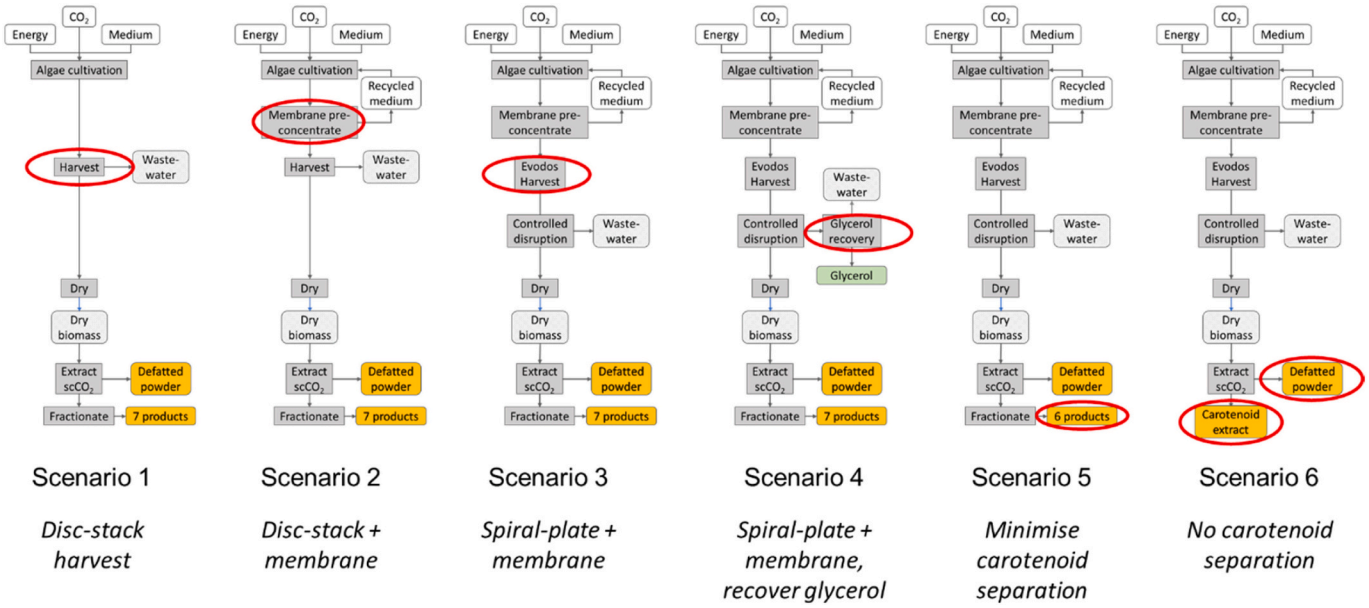

Fig. 1. Schemes to illustrate the 6 scenarios devised for sustainability assessments.

when fed to male broiler chickens. Chicks fed on diets with 0.1% defatted powder compared to those fed without showed a significant improvement in feed conversion ratio and a significant gain in weight [49]. 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.

However, realisation of the value of by-products presents several hurdles. For instance, although Dunaliella powder was granted GRAS (Generally Recognized as Safe) status [50] this does not automatically ensure European acceptance of novel food ingredients derived from this alga. The European Food Safety Authority (EFSA) defines novel foods as food that European citizens have not consumed to a significant degree prior to May 1997. It includes food from new sources, food obtained through the application of new technologies or by using new substances and the current European Union (EU) regulations on Novel Food (2015) specifically refers to food consisting of, isolated from or produced from algae.

Approval of algae-based biomass and derived extracts is currently the main bottleneck tending to reduce the potential of algae biomass as a food/nutraceutical resource. It requires complex and expensive dossiers to deliver complete characterization of independent batches of products including stability analysis and safety studies; review of the safety of the product, its source and its compounds; and evidence pertaining to Absorption, Distribution, Metabolism and Excretion. There is only a limited number of authorized algae products that have been approved and this also limits the growth of the Algae sector.

The legislation pertaining to animal feed is equally onerous: it applies principally to feed for farmed livestock, but also covers feed for horses, pets, farmed fish, zoo and circus animals, and creatures living freely in the wild and addresses the nutritional claims that can be made for certain feed products and the additives (including vitamins, colorants, flavourings, binders) authorized for use in animal feed. Regulation (EG) Nr. 767/2009: regulates All Feed Material; Regulation (EU) Nr. 68/2013: Catalogue of All Feed Material; and Regulation (EG) Nr.1831/2003: Regulates e.g. coloring agents, nutritional components currently apply to *Dunaliella*. Vitamins, provitamins and chemically well-defined substances having similar effect are however recognized (2015).

Cosmetics are covered under REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals): only chemicals that are registered are permitted for production, import, marketing and application. Biological materials such as $scCO_2$ extracts of carotenoids currently fall under the category of "substances with unknown or variable composition, complex reaction products, biological material whose chemical composition cannot identified sufficiently because the amount of ingredients is high; the major amount of the composition is unknown; and/or composition may vary significantly. A lipid-based anti-aging skin product from *D. salina* is however marketed by DSM through their acquisition of Pentapharm (Switzerland) [51].

Techno-economic, social and environmental assessments of sustainability represent important tools with which to assess new technologies for unwanted effects or potential damage of any of the technological innovations that might be introduced. When integrated, those technologies or gaps in technology that impact significantly on the sustainability of the whole are highlighted in order to allow further improvements for sustainability in a continuous cycle. The assessment is also future-oriented and recognises that innovations in technology will not be perfected within the current time frame of a given project.

Technology assessments are difficult if not impossible to carry out in an objective manner since subjective decisions and value judgments have to be made regarding a number of complex issues such as (a) the boundaries of the analysis (b) the selection of appropriate indicators of potential positive and negative consequences of the new technology. To minimise the tendency for bias by values of the most powerful stakeholders, i.e. the developers and proponents of the new technologies under consideration, the D-Factory project partners held a workshop to review technology status and evaluate findings to date. All partners were offered the chance to score technologies in an anonymous way. This also provided the opportunity for new technologies to be brought forward. From this exercise a set of 6 scenarios were developed for sustainability assessments based on integrated designs for construction of a fully operational industrial scale D-Factory facility in year 2025, and operation of a 20 Ha site producing a total algal culture volume of 993,600 m^3 (50 t AFDW powder y^{-1} or 167 kg d^{-1}), for 25 d $month^{-1}$ for 12 months i.e. 300 d y^{-1}. For this exercise, data were required. Carotenoids, lipids and glycerol comprised the product portfolio, and centrifuges, supercritical fluid ($scCO_2$) extraction, solvent extraction, liquid-liquid extraction, membranes and chromatography were the main involved technologies. Some of the main points that were taken into consideration in developing the scenarios are highlighted below, but for full details the reader is referred to [52]. The base case scenario for the biorefinery process envisaged the following steps and Fig. 1

Table 5
Overview of products and reference products [52].

Products	Market	Reference product
Polar-lipids, non-polar lipids, free fatty acids	Specialist animal feed Surfactants	Rapeseed oil
Defatted powder	Feed	Soy + cereals
Lutein	Nutraceutical	Lutein purified from marigold
Zeaxanthin	Nutraceutical	Zeaxanthin purified from marigold
Chlorophyll	Food colorant	Extracts from green plants such as spinach
All-trans β-carotene	Food colorant	Synthetic *all trans* β-carotene
9-cis β-carotene	Pharmaceutical	Standard: none (novel product)
	Sensitivity: Nutraceutical	Sensitivity: like *all-trans* β-carotene
α-carotene	Nutraceutical	Like *all-trans* β-carotene
Glycerol	Multiple	Generic substituted chemicals

illustrated the 6 scenarios that were developed for sustainability assessments:

- Evodos-type spiral plate centrifuge for harvesting cells
- Membrane technology as a pre-concentration step for harvesting cells to lower energy costs and permit effluent recycle
- Controlled cell rupture using water, which also washes biomass to remove salt
- Biomass is dried - drying step uses spray-drying
- Supercritical CO_2 ($scCO_2$) and organic solvents are used to fractionate extracts into increasingly pure preparations of high-value compounds.

The products and reference products for the assessments is shown in Table 5.

The economic assessment analysed CAPEX (fixed capital investment + working capital or working capital investment); OPEX (direct or variable production costs, fixed production costs, general expenses); products' prices based on market analysis and determined Net Present Value (NPV), the Internal Rate of Return (IRR), the Breakeven Revenue and the product contribution margin. In the absence of a market value for *9-cis* β-carotene, the price of lutein (€1470/kg) was used as a marker for working out a price equivalent, although this should increase with the expected certified health claims.

For calculation of the NPV, the net operating cash flows over the life of the project were summarized based upon the sales income and operating costs excluding all costs which did not represent a cash movement e.g. depreciation costs. The capital investments required to build the biorefinery plant was then added to this cash flow. The cash flow was then discounted at a discount rate which represented the opportunity costs of investing in an alternative project. The economic assessment used a discount rate of 5% as per EU guidelines. The NPV is then the sum of the discounted cash flows. The decision criterion for the evaluation of an investment project is that the NPV should be at least zero or positive.

The IRR is the discount rate at which the NPV is just equal to zero. The higher the IRR, the more favourable the investment project appears because it implies that future cash flows could be discounted at a higher discount rate until the NPV would become zero. An IRR of 25% is generally considered “as the threshold for securing capital investment in new processing technology”. This threshold was used as a benchmark which the D-Factory would have to achieve in order to become attractive for investors. If a given scenario failed to achieve this threshold return the increase in the product prices required to achieve this threshold return was calculated.

The breakeven revenue is the revenue required for the business to cover its costs. Breakeven revenue equals the fixed costs divided by the gross margin ratio and consequently reflects the sales required to cover both the direct costs of production and the fixed costs of operation. The business only starts making a profit once sales rise above the breakeven revenue.

2. Outcomes

Outcomes from the D-Factory are described below with reference to the 3 questions posed:

(1) Can the biomass yield of *D. salina* be increased sustainably?
(2) Can *9-cis* β-carotene be separated cleanly and in high yield from other components of harvested *D. salina* biomass?
(3) Can *9-cis* β-carotene be produced sustainably in a biorefinery with lower energy costs compared to traditional chemical processes, and reduced carbon emissions, water usage and waste?

2.1. Can the biomass yield of D. salina be increased sustainably?

A coastal D-Factory pilot with geo-textile lined OPRs fitted with paddlewheels, sensors and monitor was established (Fig. 2) [2].

These held sea- and salt-water maintained at 10–15% salinity to cultivate the halophytic algae and liquid pressurized CO_2 was bubbled in for carbonation for algal growth. Algae were harvested by partially or completely draining the raceways using centrifuges and the biomass was stabilised by spray-drying techniques. The OPRs were operated for 300 days per year, in winter (5 °C–15 °C) and summer (> 40 °C) and wastewater containing high salt loads and organic matter was 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. Artemia were controlled with traps and with modulation of environmental parameters including increase in salinity: to allow continuous summer production the salt concentration was increased to above 18%. On the other hand, protozoa infestation was not as easily controlled; protozoa are common in all marine salts

Fig. 2. A coastal D-Factory pilot facility set within the NBT (Ltd) Production site in Eilat, Israel. Lined raceways 150 m long x 4 of 5 m width, fitted with paddlewheels, hold sea- and salt- water to a depth of ~20 cm. Local strain, *Dunaliella bardawil*.

and in all ocean or marine waters. They come as cysts and cannot be eliminated; the only protozoa-free marine salt available is costly, kiln-dried salt.

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: 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}. Dried biomass was stable for at least 1 year, stored in the dark at −18 °C, in food-grade polyethylene bags held under vacuum. Biomass could therefore be harvested, washed free of salt on site for reuse, and dried to a powder and batches of the powder could be stored and blended before extraction to reduce seasonal variation in biomass quality.

The knowhow to cultivate natural strains of halophytic *Dunaliella* in OPRs was transferred to a second inland facility [2], which offered the opportunity to assess the use of flue gas CO_2 for carbonation for algal growth and the use of salt from an underground salt-mine. Flue gas CO_2 was bubbled from natural gas combusted in a local 14 MW CHP plant (up to 7% CO_2), which also supported a heating system designed for temperature control in winter. Furthermore, the salt mined from the underground caverns was protozoan cyst-free and treated wastewater could be injected back into empty underground salt caverns.

Cascade raceways (capacity 15m^2, 525 L) were also tested but achieved only a marginally better biomass productivity of 4.4 g m^2 d^{-1} in pilot scale and were heavily dependent on inputs of fresh water to compensate for evaporative losses which, using thin (4 cm) layers of culture impacted significantly on salinity [2].

For development of high density inoculum, tubular PBR's were tested: these should increase volumetric productivity because a shorter light path length can be applied. However, they rapidly became fouled when algal glycerol leaked from the wall-less algae and supported heterotrophic growth of halotolerant bacteria, yeasts and other fungi. Increasing the salt concentration as a measure to control predators also increased the glycerol content and the attendant production of biofilms which were difficult to clean from the insides of the tubes without the availability of *in situ* technology. Flat-panel PBRs however, supported good growth of orange carotenogenic *Dunaliella* for inoculation and the plastic bags could be discarded once contaminated.

Under continuous light with non-limiting CO_2, biomass yield increased but was not sustainable because photosynthetic and respiratory systems of the algae were damaged [53].

The sustainability assessment concluded that savings of up to 90% of greenhouse gas emissions and other environmental impacts, compared to the state of the art, seemed achievable over the next years for *Dunaliella* biomass production. Specific findings included the following (see [54] for full details):

- On-site solar power or renewable steam production from geothermal energy at certain locations could significantly decrease fossil-based energy input and related GHG emissions - up to almost zero.
- Fresh water (or seawater) use may be reduced with efficient medium recycling, reducing costs and environmental burdens.
- Wastewater treatment requires land, but the amount of treatment needed may be minimised by harvesting cells intact.
- Raceways require complete ground sealing and could be sited on sealed, disused, industrial brownfield sites instead of agricultural land.
- CO_2 from the combustion of natural gas direct flue gas injection does not need flue-gas treatment and does not impact on FDA or regulatory approval for algae-derived products. However, if the decarbonisation policy direction initiated today is successfully implemented in the coming decades, increasingly few CO_2 sources will be available for CO_2 utilisation from exhaust gases in the future.
- Integration of an algal biorefinery with existing facilities for producing salt products or seawater desalination facilities minimises the environmental burdens of energy and material use, impacts on local freshwater availability and disposal of saltwater as well as efforts for regulatory compliance.

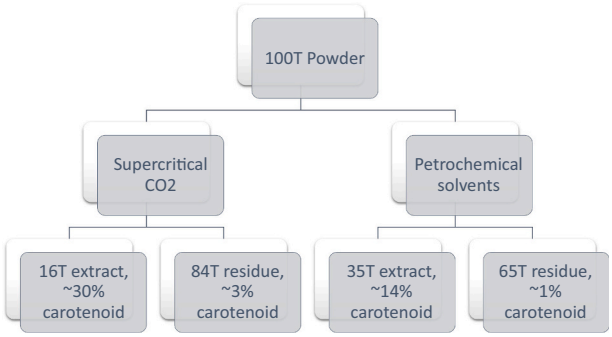

Fig. 3. Schematic to illustrate the effect of extraction with $scCO_2$ compared to the use of non-polar and polar petrochemical solvents on the yields of extract and defatted powder (residue) and concentration of carotenoids.

2.2. Can 9-cis β-carotene be separated cleanly and in high yield from other components of harvested D. salina biomass?

$scCO_2$ technology requires algal biomass in powder form for processing, so shipping powders to industrial scale $scCO_2$ facilities, which are CAPEX intensive, overcame a logistical hurdle. However, lyophilisation was too costly for industrial-scale purposes. Spray-drying technology offered a lower cost solution, provided the particle size was adjusted to suit $scCO_2$ technology, since fine (100-300 μm) free-flowing powders proved to be too powdery for the flow-through of $scCO_2$, and caused blockages in the extractor. Spray-dried particle size is, however, adjustable to meet size criteria needed for $scCO_2$ extraction.

Using lyophilised powders of *Dunaliella* biomass, the use of $scCO_2$ yielded enriched extracts of high-value carotenoids for formulation testing and defatted powders for by-product development (Fig. 3). 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% were hydrophobic carotenes. *9-cis* β-carotene was particularly enriched in carotenoid extracts (*9-cis* β-carotene: *all*-trans βC ~1.5). However, ~50% of the oil remained in the defatted powder and included 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.

Using petrochemical solvents, polar and non-polar combinations of solvent (methanol or acetone followed by hexane; MTBE-methanol) extracted almost all carotenoids present in biomass (91%), but extracts were not as enriched in carotenoids as for $scCO_2$ extracts. Extracts typically represented ~35% by weight of the total material and contained ~14% total carotenoids.

From results of further fractionation to obtain increasingly enriched preparations of individual carotenes and xanthophylls based on combinations of solvent, counter current chromatography (HPCCC) and preparative HPLC technologies, and the use of solvent resistant membranes to remove solvent, an integrated purification strategy was devised with the aim of scale-up.

Separation challenges were solved in analytical and laboratory-scale separations but proved extremely challenging in scale-up. The purified carotenes were also unstable to light and oxygen and increasingly unstable on purification from lipids. *9-cis* β-carotene could not be separated from other carotenes using HPCCC due to their similar partition coefficient in the range of solvents systems tested. Instead, a strategy using preparative HPLC was developed, whose success ultimately depended on the nature (concentration of *9-cis* β-carotene) in the starting material. Separations were characterized by low product yields, long

run times and low loading capacity (1.8 g sample for a 50 mm diameter column). Purified preparations of *9-cis* β-carotene could be obtained which represented 94% of total carotenoids detectable using a photodiode array detector, but only 82% *w*/w of the dry matter because of the presence of lipids which formed an apparent super-structure with *9-cis* β-carotene. These preparations were stable for up to 3 months at 4 °C in petrochemical solvents but degraded readily in the presence of water containing oxygen and light.

HPCCC and HPLC technology were difficult to model quantitatively for the sustainability assessment because important experience from operation at scale was not available. An analysis of the environmental impacts, however, clearly showed that the full sequence of downstream processing steps would impose enormous burdens, dominating climate impacts of the whole life cycle by far, with the largest emissions coming from preparative HPLC. Alternative scenarios which produced fewer products and delivered *9-cis* β-carotene as a mixture, saved energy and solvents.

2.3. Can 9-cis β-carotene be produced sustainably in a biorefinery with lower energy costs compared to traditional chemical processes, and with reduced carbon emissions, water usage and waste?

From the economic assessment of sustainability, improvement of productivity in the ponds from 3 to 5 g m^{-2} d^{-1}, could be expected to generate a 27% profit margin, provided the costs of production of *9-cis* β-carotene could be lowered and the market for *9-cis* β-carotene products could be developed. Costs of production of *9-cis* β-carotene were excessive because of the technological difficulties that arose in separating the β-carotene isomers using HPLC.

The main source of biorefinery waste was identified as glycerol, soluble protein and lipid arising from cytosolic cell biomass released from ruptured cells harvested using Westfalia-type disc stack centrifuges. Before discharge to water bodies wastewater effluents require treatment to meet local legislation. High salt effluents are corrosive and the number of active bacterial species for aerobic treatment is restricted to halophiles, which precludes anaerobic digester treatment at high salt concentrations. Depending on land availability and capital investment, an aerobic treatment system combined with a membrane bioreactor to remove suspended solids and chemical oxygen demand (COD) was recommended. Wastewater effluents may alternatively be reused if harmful organisms can be destroyed or removed along with any inhibitory chemicals. Table 6 provides a summary of waste treatment options.

Wastes could be minimised: harvesting cells intact using Evodos spiral plate centrifugation offered the opportunity to recover enzyme and polar lipopolysaccharide by-products, as well as reduce costs of effluent waste management. However compared to use of a disc-stack centrifuge, which harvested biomass at 12,000 L h^{-1} spiral plate centrifugation caused losses of *9-cis* β-carotene due to overheating and long processing times. The Evodos T50 continuous harvesting prototype harvested cells at 2500 L h^{-1}, with 95% separation efficiency, > 20 h d^{-1} and pastes comprised of up to 40% solids with cells ~90% intact. The Evodos batch-type T10, harvested cells optimally at 350 Lh^{-1}, with 95% separation efficiency (20–30% solids in pastes with cells > 95% intact). Evodos-harvested powder was also less-easily processed using $scCO_2$ due to the presence of salt in the unwashed lyophilised biomass. A two-step approach which integrated an ultrafiltration polysulfone membrane unit with Evodos spiral-plate centrifugation was tested to see if this could offer improvements in minimising waste. Based on a concentration factor of 5 and an average permeate flux of 22 L m^{-2} h^{-1}, a reduction of the OPEX + CAPEX of 52% and reduction of energy consumption of 66% could be achieved using the two-step approach compared to the use of the spiral-plate technology alone [55,56]. However, the more fragile carotenogenic cells now tended to rupture. Downstream processing with supercritical CO_2 will generate a small loss only of CO_2, because most is recovered by condensation and re-used in the process. Wastes from downstream processing with solvents (HPLC, HPCCC) can be minimised by implementing solvent recycling combined with the use of membranes to recover solvents.

Table 6
Available wastewater treatment options.

Option	CAPEX	OPEX	Suitable for recycle/re-use	Suitable for discharge
Non-oxidising biocide	+	+ +	No	No
Membranes	+ + +	+	Yes	Yes
Ozone (or Peroxide) Low salinity	+	+ +	Yes	No
Ozone (or Peroxide) High salinity	+ +	+ + +	Yes	No
Chlorine	+	+	Yes	No
Chlorine dioxide	+	+ +	Yes	No
Peracetic acid	+	+ +	Yes	No
Aerobic treatment	+ +	+ +	Yes	Yes
Anaerobic treatment (of sludge)	+ + +	+	No	No

3. Going forwards

Biobased production of *9-cis* β-carotene by cultivation of the photoautotrophic, halotolerant microalga, *D. salina* could deliver extraordinary increases in resource efficiency and environmental impact reductions, compared to energy- and resource-intensive chemical synthesis of *9-cis* β-carotene and delivers preferred qualities compared to synthetic forms. However, the technology for downstream processing still requires development.

Significantly, costs could be reduced if $scCO_2$ extracts were further enriched in *9-cis* β-carotene. Two routes may be envisaged:

3.1. Use of strains selected for high relative content of 9-cis β-carotene

Under controlled laboratory conditions algal strain was identified as the most important factor influencing carotenoid production. Mutants that were developed in the laboratory using a classical mutagen, 5-Bromouracil, when tested outdoors in OPRs were either unable to compete with prevailing natural strains or to withstand environmental conditions. By contrast all D-Factory strains of *Dunaliella* that were isolated from natural environments were cultivated successfully in small scale from the laboratory to medium size 100 m^2 OPRs. These data imply that the most practical approach for tailoring cultivation at scale would be one that was based on cultivating locally sourced hypercarotenogenic strains selected for high *9-cis* β-carotene.

In the D-Factory 27 new monoclonal *Dunaliella* strains were isolated from saline waters and preserved successfully using new cryogenic methods, despite the absence of a cell wall and presence of delicate flagellae. They are now catalogued in the D-Factory reference library and available to the general public by application to the Marine Biological Association 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 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 [57,58].

3.2. Cultivation with red light

The problem of variable microalgal biomass quality with low *9-cis* β-carotene content might be circumvented by developing low-cost, controlled light-emitting diode (LED)-based lighting for use in OPRs, because *D. salina* will increase the content of *9-cis* β-carotene relative to *all-trans* β-carotene 3.5-fold and simultaneously increase the total

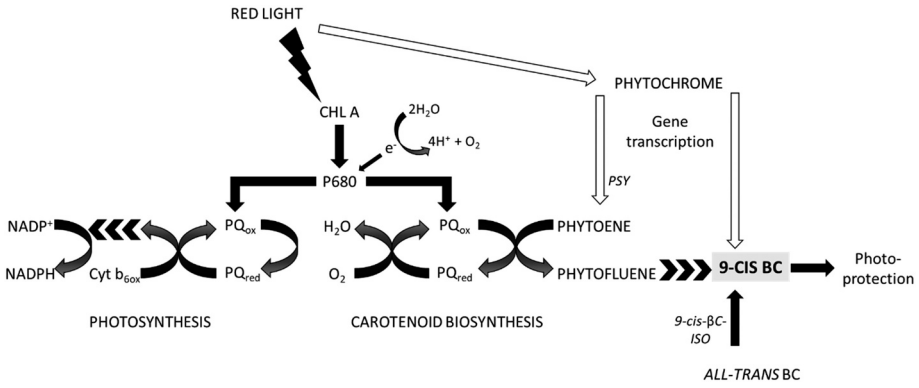

Fig. 4. Regulation of the pool size of *9-cis* β-carotene. Red photon flux intensity controls the partitioning of electrons either for carotenoid biosynthesis or for photosynthesis, via energy absorption by chlorophyll and the PQ pool. Red photon flux also controls phytochrome regulation of the production of gene transcripts for phytoene synthase and β-carotene isomerases. CHL A: chlorophyll a; P680: chlorophyll a, primary electron donor of Photosystem II; PQox: plastoquinone, oxidised form; PQred: plastoquinone, reduced form; Cyt b6ox: cytochrome *b6f* complex, oxidised form; NADP+ NADP oxidised form; NADPH: NADP reduced form; PSY: phytoene synthase; 9-cis-βC-ISO: 9-cis βC isomerase. [From Ref [59,60]].

content of carotenoids in biomass when cultivated under red light (625–680 nm). The absolute amount of *9-cis* β-carotene increases ~5-fold, without requiring nutrient limitation, sub-optimal temperatures or excessive salt concentrations [59–61]. The effect of an increase in *9-cis* β-carotene is evident even under very low intensity red light (ca. 10–17 μmol m^{-2} s^{-1}). Red light appears not only to support the growth of *D. salina* but also to upregulate the entire biosynthetic pathway of carotenoids, and seemingly involves chlorophyll *a*bsorption of red light photons coupled to oxygen reduction and phytoene desaturation with a plastoquinol:oxygen oxidoreductase, as well as the upregulation of phytoene synthase by the red light photoreceptor, phytochrome, which may also control β-carotene isomerase to increase the rate of conversion of extant *all-trans* β-carotene to *9-cis* β-carotene [60,62] (see Fig. 4). In this regard, recent evidence shows that *15-cis* phytoene over-accumulates with red light and with the use of herbicides and is likely converted to *all-trans*- and *9-cis*-β-carotene via *9,15-di-cis* phytofluene by a series of isomerisation and desaturation reactions [62].

4. Conclusions

Valorisation of the efficacy of *9-cis* β-carotene in treating atherosclerosis, psoriasis, inhibiting atherogenesis and retinitis pigmentosa is becoming increasingly urgent. We have estimated that the sale of *9-cis* β-carotene oil extracted from *Dunaliella* biomass could generate an income exceeding 30 million € p.a. Furthermore, after extraction of the *9-cis* β-carotene oil, the remaining defatted biomass offers a potential source of protein/carbohydrates, which could be more effective for food/feed applications compared to the corresponding synthetic variants and offer large opportunities to formulate new types of feed products. The worldwide production of feed is 1 billion tonnes per year (International Feed Industry Association, www.ifif.org), and consequently the potential added value for EU/world is high.

Sustainable low cost production of *D. salina* using improved pond designs with flue gas CO_2 combined with heating/cooling for all-year-round cultivation at optimal temperatures and use of high-quality salt, as described by the D-Factory biorefinery project, shows the potential for at least doubling the levels of productivity normally achieved in industrial-scale cultivation, whilst savings of up to 90% of greenhouse gas emissions and other environmental impacts, compared to the state of the art, seem achievable over the next years.

Use of high density inoculum enriched in high *9-cis* β-carotene strains for *D. salina* combined with development of new LED-based *D. salina* cultivation technology suited to OPR cultivation offers further potential in cultivation for delivering reproducible, low-cost bio-based production of *D. salina* biomass, with a consistently high relative content of *9-cis* β-carotene. This could alleviate to some extent the techno-economic and environmental problems identified with its downstream purification. Realisation of these findings will require active engagement with multiple stakeholder groups, over several years, as well as larger-scale projects to develop the technologies and collate the data needed to support acquisition of a Medicine Licence to prepare the market for entry of novel *9-cis* β-carotene products.

Author contribution

P.H: Conceptualization, Writing—Original draft preparation, Visualization, Funding acquisition. A.B: Validation, Review and editing. P.H and A.B acknowledge critical reading from Paul Lucas, D-Factory project manager.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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