ISSN 1550-9087
VOLUME 15 NUMBER 1
FEBRUARY 2019

Industrial Biotechnology

The journal of biobased innovation to drive the global bioeconomy

Dark fermentation of photosynthetic microalgae

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Catalyzing Innovation

Brewing Change: Dark Fermentation of Photosynthetic Microalgae

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Abstract

Microalgae are commonly viewed as being photosynthetic, requiring light for growth. However, certain algae can grow much more efficiently in the dark, in enclosed tanks, when provided hydrocarbons like sugar or organic acids. This facultative ability of some photosynthetic algae to switch to a dark aerobic fermentation process results in bioactive or functional compounds being produced in a fraction of the time and using a fraction of the water otherwise required for conventional photosynthetic production. Stainless steel tanks—like those used for yeast culture in beer breweries—can be coupled with sophisticated control systems and strategies for the precision cultivation of specialty microalgae cells. The ''craft brewing'' approach has proven a commercial success for a few species and target compounds. Extending this approach to additional photosynthetic algal species could provide advantages over current practices and sources of raw materials. This overview describes our company's use of dark aerobic fermentation for the manufacture of whole-cell and extracted raw material from carotenogenic microalgae. One outcome is an industrial process adopting more resource-friendly practices required for consumer acceptance in certain market applications. A second outcome is a path to supplying sustainable products to replace chemically synthesized products in markets where it appears reasonable to do so. In addition, methods are being discovered for the production of innovative compounds for use in personal care products and other formulations. Broad application of the technology will succeed through engagement with corporate partners committed to sustainable production and products.

Introduction

Sustainability through industrial biotechnology plays a fundamental role in achieving the vital UN Sustainable Development Goals for the 21st century.[1] A key element of this plan is innovation in the design of industrial production processes. This can entail shifting a current manufacturing practice toward becoming more resource-friendly, and by incorporating economic efficiencies to facilitate substitution of non-renewable fossil-fuel based chemical products with renewable biological sources.[2]

This aligns well with the increasing demand by consumers for plant-based ingredients of certifiable origin. Plant-based diets are rapidly gaining traction, especially in Europe and Asia, with strong projections through 2021.[3] However, the willingness to pay for sustainability in food by consumers still requires meeting general expectations for price and quality.[4] For the health and personal care sectors, including cosmetics, the growing trend for ingredients being sustainable and natural[5,6] also reflects the lingering uncertainty about long term health effects from petrochemically-derived compounds such as synthetic colorants and supplements.[7–9]

Kuehnle AgroSystems (Honolulu, HI) has long held an interest in plant and algal carotenoids and other chromophores as sustainable ingredients. Recently, its approach towards research in new raw materials shifted to include developing and integrating transformative manufacturing. While the company's current focus is on skin care ingredients, the outputs of its patented and patent pending technologies have application across several market sectors.

Sustainable Carotenoids

Carotenoid pigments are one output target for innovative industrial production processes. The global carotenoids market was valued at about US$1.6 billion in 2017, with projections of reaching US $2.1 billion by 2025.[10] Carotenoids are long, double-bonded, carbon-chain molecules of high commercial value with well-established colorant, antioxidant, anti-inflammation, anti-cancer, and photoprotective properties when ingested or applied topically. They include the carotenes (the unoxygenated carotenoids such as β-carotene, α-carotene, and lycopene) and the xanthophyll carotenoids (such as astaxanthin, lutein and its isomer zeaxanthin, canthaxanthin and fucoxanthin). Carotenoids are used across the industries of feed, food and personal care (e.g., pharmaceutical, cosmetics and nutritional supplements). They also find application in the synthesis of flavors and fragrances,[11] in pet food and endurance animal diets,[12,13] and have potential in electronics applications such as for artificial photosynthesis and photoelectric devices.[14,15]

While many carotenoids are naturally sourced from plants and microbiota,[16–18] several are manufactured primarily by chemical synthesis using carbon building blocks derived from petrochemicals. These include synthetic astaxanthin, canthaxanthin, β,β-carotene (Provitamin A), β-apo-8′- carotenal, β-apo-8′-carotene, lycopene and citranaxanthin.[19] There is chemical non-identity between some biologically derived and synthetic carotenoids such as astaxanthin. Chemical synthesis of carotenoids offers the advantages of supplying very large volumes, reduced cost, and elimination of geographic and seasonal restrictions when compared to sourcing from photosynthetic microalgae and plants. Synthetic carotenoids accounted for 76%

DOI: 10.1089/ind.2019.29159.aku

of the worldwide carotenoids market in 2014. For perspective, about 97% of the astaxanthin volume and about 70% of β-carotene volume on the market is synthetic. The consumer push for greater sustainability is driving strong growth of the natural carotenoids market, forecast to increase at 4.2% annually from 2018 to 2025.[10]

The lead carotenoid in market value is astaxanthin, due to its extensive use in fish and animal feed. As a fish feed additive, predominantly synthetic astaxanthin is used to elicit pink flesh and promote growth of farmed salmonids (salmon and trout), red sea bream, and others; without it, the fish flesh is grayish white and viewed negatively by consumers. The synthetic colorant sells at about one-third to one-sixth the price of natural algal astaxanthin. Even so, the additive comprises around 10–15% of the total feed cost,[20] which can make shifting to natural sources a challenge while meeting consumer expectations.[21,22] Synthetic additives to feed, as well as to food, are regularly reviewed as to maximum permitted concentrations to ensure consumer safety (for example, see the 2002 European Commission Opinion for canthaxanthin),[23] and labeling regulations apply such as the U.S. FDA requirement for ''color-added'' to farmed salmonids[24] for transparency.

Natural astaxanthin is used primarily in nutraceuticals, skin care products, and specialty sectors such as feed ingredients for pets or performance and show animals. For natural astaxanthin, the microalga *Haematococcus pluvialis* (synonym *H. lacutris*) is a preferred production microorganism due to very high total carotenoids accumulation potential. [25] *H. pluvialis* is cultivated photosynthetically, as is its cousin *Dunaliella*—a commercial source of natural β-carotene—within the taxon Chlamydomonadales. The slow-growing photosynthetic *H. pluvialis* is particularly susceptible to crop failure due to contamination or predation, and it requires a lengthy period of exposure to high light (photoinduction) and controlled nutrient depletion to produce pigment-rich biomass.[26]

Astaxanthin and other carotenoids serve a photoprotective role in photosynthetic organisms. In the algal cell, the molecules are photoprotective in three different ways: by filtering light (i.e., absorbing harmful radiation), by quenching excess energy to prevent formation of reactive oxygen species, and by scavenging (reacting with) free radicals to mitigate cell damage.[27] Astaxanthin and β-carotene perform all three functions of filtering, quenching, and scavenging in the cell. Lutein is considered both a quencher and free radical scavenger, while zeaxanthin primarily performs a scavenging role.[28] These functionalities are largely preserved for carotenoids *ex situ* when consumed or applied topically and thus are valued in nutrition and in skin care formulations, as reviewed elsewhere. A combination of all the major carotenoids in a single natural ingredient developed by Kuehnle AgroSystems for consumption and for personal care products (described below) offers a multifunctional approach to combatting skin damage and associated skin aging, inflammation and discoloration. Consumption of a diet containing carotenoids also contributes to fitness. In fish, this improves numerous metrics contributing to health, disease resistance, survival rate, and reproductive performance.[29]

One well-known life cycle of *H. pluvialis* involves the stages of cellular differentiation from green vegetative macrozooids into red aplanospores (haematocysts or cysts). Mature cysts have a thick, rigid cell wall that prevents the extraction of carotenoids without added drying and milling (cracking or rupture) steps prior to extraction.[30] Extraction costs are significant contributors to operational expenses for the cultivation, harvest and extraction phases for photosynthetic *H. pluvialis.*[31] This cell wall also prevents the direct use of cysts in feed formulations as single-cell colorant (abbreviated SCC, akin to single-cell protein SCP). Due to poor digestibility and thus low bio-accessibility, the algal cysts need to be pre-ruptured for feed formulation; this is similar to the pre-rupture needed for red yeast cells.[32,33] Pre-rupture significantly lowers the shelf-life of biomass to only a few months.

Fermentation Production of Microalgal Carotenoids from *Haematococcus pluvialis*

The aim of scientists at Kuehnle AgroSystems was to reconfigure industrial production processes for photosynthetic microalgae, starting with the Chlamydomonadales, as a source of sustainable functional or bioactive targets. For production of carotenoids, the company's solution employs dark aerobic fermentation (heterotrophy) for high-pigmented biomass. For heterotrophic production of normally photosynthetic species, such as *H. pluvialis*, light energy is substituted by a supply of organic acids to drive biochemical processes.

Among various production scenarios, fermentation is considered the most economical and scalable method of algae production in a detailed model.[34] Heterotrophic production of microalgae has found commercial success primarily for lipids, protein, and polysaccharides including beta-glucan, but less so for pigments in sufficient yields such as for *Chlorella*.[35] The company's researchers discovered that heterotrophic *H. pluvialis* is an excellent microbe for production of carotenoids using vegetative motile pigmented macrozooids, with no extraction or digestibility constraints from a rigid cyst wall that forms during hypercarotenogeneis in photosynthetic production. Unlike current photosynthesis-based approaches and previous bench-scale attempts at heterotrophy with this species, Kuehnle AgroSystems' approach overcomes extremely low specific growth rates, low specific productivity, low cell density, and eliminates the usual photoinduction and lengthy carotenogenesis with obligate encystment for *H. pluvialis.*

For the carotenoid pigments, this approach has eliminated manufacturing risks presented by photosynthetic production, reduced water usage as a sustainability metric, and eliminated encystment and milling, all with an eye to narrowing the cost gap with chemically synthesized carotenoids.

The Kuehnle AgroSystems patent-pending process for rapid generation of biomass with hypercarotenogenesis proceeds under complete darkness for the entire production cycle (see cover illustration). This pivot away from photosynthesis enables moving operations indoors into a ''brewery'' setting, using steel tanks for rapid, high yields of biomass for extraction or for SCC. Additionally, it provides an efficient scale-up propagation method suited to integration with existing photosynthetic operations. Regulating the precise cultivation conditions and thus the cell metabolism in new ways using fermentation allows for modifying the cell composition. This includes the values for the carotenoids astaxanthin, canthaxanthin, lutein/zeaxanthin, and carotene, as well as the protein, lipid, and carbohydrate values.

Using a strategy of dark fermentation for *H. pluvialis* eliminates the variable of photon absorption rates that determines photosynthetic biomass concentration. This means that a production process can operate with cultures at much higher cell density and lowered water usage. Reduction of the water footprint (consumption and mass-energy flows) is identified as a key determinant of cost savings for current production methods of *H. pluvialis*.[31] Production using a hybrid photobioreactor and open raceway pond system is calculated to require over 3,700 L of fresh water to produce a single kilogram of photosynthetic algal biomass. In comparison, fermentation cultivation using organic acid feedstock may reduce water requirements by a significant one to two orders of magnitude, estimated at less than 40 L per kilogram biomass. Use of a non-sugar carbon input helps address the water resource issues surrounding glucose as a water-intensive feedstock commonly used in microbial fermentations.[36]

Meeting the challenge of production by fermentation at Kuehnle AgroSystems resulted in the following advantages:

- vastly accelerated cropping cycles compared to photosynthetic production,
- significant increases in volume capacity and reduced water consumption,
- flexibility to integrate into existing photosynthetic operations,
- surpassing the minimum metrics modeled for economic manufacturing of pigment from *Phaffia* yeast,[37]
- ability to use non-sugar feedstocks and non-genetically engineered strains,
- repackaging of natural colorant and supplement into a digestible whole-cell form,
- and discovery of innovative compositions with proven applications.

Additionally, the controlled manufacturing process is highly efficacious for the highest quality ingredients from *Haematococcus* and other Chlamydomonadales, offering these benefits for marketing and distribution partners:

- high supply chain confidence,
- GMP for personal care, food, and beverages,
- no heavy metals or contaminants,
- rapid turnaround,
- consistent pricing, and
- traceable and renewable.

Applying the Technology

There are multiple opportunities for whole-cell and extracted raw materials from heterotrophic production of *Haematococcus* to address new market needs across value chains (*Fig. 1*). Some examples are given below. The company recognizes that applying the technology will be successful through active engagement with corporate partners who have the means and the responsibility to shepherd sustainable production and products into the future.

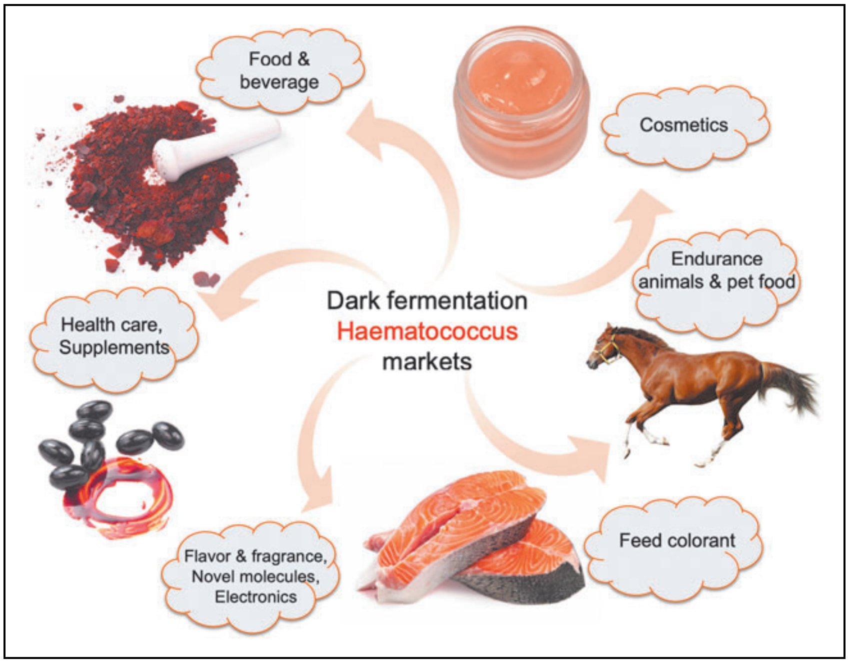

Fig. 1. Whole-cell and extracted raw materials from heterotrophic *Haematococcus* and other algal species address new market needs across value chains. Such raw materials offer options with numerous new applications, branding benefits, and a broader spectrum of product formulations than is possible through use of synthetic molecules.

ASTAFUSION™ OLEORESIN FOR COSMETICS

An immediate focus of the company has been on cosmetic applications. Development of new skin care formulations for the sophisticated consumer relies on identification of suitable raw materials with documentation of efficacy. Major targets for efficacy involve anti-aging benefits such as preventing collapse of the skin extracellular matrix that causes fine lines and wrinkles, moisturizing, and balanced skin tone. One specific anti-aging target in the skin is the carbonylated proteins. These are oxidized proteins synthesized through the reactions of lipid peroxidation. They are observed in both the epidermis and dermis of skin that is chronically exposed to sunlight.[38,39]

One raw material for skin care purposes being produced commercially by the company is called AstaFusion™. Derived from heterotrophic biomass, it is rich in a mixture of carotenoids complexed with nourishing essential fatty acids. About two-thirds of these lipids comprise omega-3, -6, -7, and -9 unsaturated fatty acids, including linoleic reported to induce beneficial skin cell autophagy and omega-7 fatty acids purported to support skin health. For this ingredient, harvested heterotrophic *Haematococcus* macrozooid cells are dried, extracted with a cosmetically acceptable solvent to obtain a dark red-orange oleoresin, which is then purified and solubilized into skin-compatible oils or other vehicles to document efficacy and to develop formulations. Recent rigorous testing using human melanocytes and dermal fibroblasts, undertaken by Nikko Chemicals (Tokyo) and Tokyo University of Technology, demonstrates the potential of this raw material to even skin tone and promote the stability of the dermal matrix under stress conditions via multiple paths. AstaFusion™ inhibited tyrosinase biosynthesis, prevented the progression of photoaged dermis due to carbonylated proteins, and suppressed expression of MMP-1 and IL-8 mRNA, which otherwise promote the degradation of the extracellular matrix (Yamawaki et al., manuscript in preparation). The high bioactivity of the ingredient offers a relatively low inclusion rate for a range of hues such as gold and peach, depending on the cosmetic base.

AQUAZANTHIN™ WATER-SOLUBLE CAROTENOIDS

Kuehnle AgroSystems scientists discovered compositions of heterotrophic algal water-soluble carotenoids that are heat-stable, color-stable, and demonstrate photoprotective properties (patent pending). Proprietary processing of algal biomass cultivated at pilot scale in complete dark fermentation revealed water-soluble carotenoid compounds that include multiple xanthophyll moieties. (It should be noted that ''water-soluble'' is distinct from ''water-dispersible,'' which pertains to coated lipophilic oleoresin preparations.) The company's new cosmetic water-soluble ingredient, called AquaZanthin™, has promising UV light absorption, antioxidant, and anti-free radical reduction capacities. This pioneering development of stable water-soluble carotenoid ingredients opens up the possibility to formulate in the water phase of a range of food, beverage, eye care, skin care and other future products.

WHOLE-CELL INGREDIENT FOR COSMETICS

Tested as a whole-cell cosmetic ingredient at a very low use rate, the unextracted and unmilled biomass can naturally colorize a cosmetic base through releasing bioactives and add visual effects as valued components of wash-off facial scrubs and masks. Both the extracted and the whole-cell cosmetic ingredients are stable in freeze-thaw testing, and retain their color, odor and appearance over a storage period standard for the industry. Produced by this method of manufacture, they also conform to cosmetic ingredient requirements for microbial load and heavy metals.

Fig. 2. Ornamental fish, such as these female long fin cherry barbs, show enhanced color in fin and body markings within days of being fed a diet with whole-cell algae. The diet consisted of heterotrophic macrozooid *Haematococcus* as single-cell colorant (SCC) that is naturally complexed with omega-3 fatty acids. This was incorporated into a basal diet of TetraMin® XL Tropical Flakes. A treatment fish is on the right, a control fish fed basal diet only is on the left. Coloration serves as an indicator of digestibility of thin-walled whole cells (macrozooids) without a cell pre-rupture step.

SINGLE-CELL COLORANT FOR FEED, DIETARY SUPPLEMENT

Red motile macrozooid *Haematococcus* containing commercially relevant total pigment content offers an alternative and new delivery system for carotenoids plus lipids and protein: digestible thin-walled whole cells. This raw material eliminates the high costs associated with the milling and extraction processes and equipment required for cysts. The company envisions that *H. pluvialis* and other similar algae, already with GRAS status, may be packaged and delivered as a dietary supplement much in the same way as whole-cell dried *Spirulina* (*Arthrospira*).

Digestibility of the raw material was verified by Kuehnle AgroSystems' researchers based on coloration of ornamental fish. Whole-cell red macrozooid *Haematococcus* produced by fermentation was formulated into a basal diet. When fed to fish, the result was intensified pigmentation of the scales and fins compared to control fish (no SCC). This preliminary evaluation in advance of aquaculture feeding trials also indicated bioavailability of the pigments from intact macrozooid biomass as single-cell colorant (*Fig. 2*). Skin coloration is a prime factor affecting the evaluation of overall quality in the fish ornamentals trade, while both flesh and skin coloration are associated with freshness and quality of food fish.[40] Numerous scientific studies attest to the contribution of exogenously supplied carotenoids in fish feed to enhanced growth in ornamentals and aquaculture. Therefore, the company sees promise in this application of the technology through interested partners.

OTHER APPLICATIONS

The precisely controllable conditions of fermentation that Kuehnle AgroSystems' researchers have established for the Chlamydomonadales allows for manipulation of high protein and starch contents, and without the need for genetic engineering. This has utility for a variety of emerging market applications, as well as appeal for integration into biorefineries for targets requiring high volume manufacturing.

Future Outlook

Biotechnological discoveries that are sufficiently disruptive are key enablers for transitioning to a biobased economy. For the xanthophyll carotenoids, a shift to dark fermentation increases output and lowers water consumption, eliminates numerous manufacturing and supply chain risks, produces a non-seasonal, high-quality material, and offers novel compositions. This approach shows promising potential for further improvements coupled with continued investment in securing scaled-up manufacturing and related areas of development being considered to boost economics.

The future holds opportunity to source additional sustainable carotenoids from multiple promising microbes,[41–43] assuming they conform with safety certifications accepted by international regulatory bodies, have compositions suited for stable formulations, and demonstrate efficacy. However, for business strategies focused on drop-in ingredients, be it un-extracted single-cell colorant or extracted carotenoids, a *Haematococcus*-based source currently remains the most viable across markets.

These examples of work by Kuehnle AgroSystems illustrates the manufacturing of commercially relevant natural products from microalgae under an improved paradigm employing more resource-friendly practices. Adoption now of such technologies by key suppliers committed to tackling the global environmental challenges, while meeting consumer preferences, will help ensure the quality of life for future generations.

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