

Research and Commercialization Feasibility Proposal

Re-Evaluation of Botryococcus Hydrocarbon Production

Heterotrophic cultivation, high-hydrocarbon strains, and recoverable productivity as the decision metric

A proof-of-concept program to test whether historically demonstrated dark cultivation of Botryococcus, later-identified high-hydrocarbon strains, modern fermentation control, and low-cost carbon streams can be combined. The purpose is not to assume commercial viability, but to run a limited feasibility study that tells us whether the combination deserves pilot-scale work.

Executive Summary

This proposal grew out of a simple observation while reviewing older algae-biofuel work: two promising lines of research appear to have developed separately. Solazyme demonstrated that selected Botryococcus braunii strains could grow in darkness on fixed carbon. Years later, other researchers identified strains with much higher extracellular hydrocarbon fractions. The obvious question is whether those findings can be combined.

B. braunii is unusual among candidate biofuel organisms because it can devote a large fraction of its biomass to long-chain hydrocarbons, including material excreted into the colonial extracellular matrix. Its longstanding weakness is slow volumetric productivity. A high hydrocarbon fraction has little commercial value if too little biomass can be produced per liter per day.

Solazyme, working under NIST Cooperative Agreement 70NANB7H7002, reported dark growth on fixed carbon in strains including UTEX 572, UTEX 2441, and N-836. The work appears in U.S. Patent 8,278,090 B1, granted 2 October 2012, and divisional U.S. Patent 8,956,852 B2. Both later lapsed for nonpayment of maintenance fees. Their lapse is useful to this research effort, but it is not by itself a freedom-to-operate determination.

Later photoautotrophic comparisons found much larger extracellular hydrocarbon fractions in other strains. In one milking-oriented ranking, Bot22, Showa, and CCAP 807/2 contained approximately 51.6%, 49.3%, and 39.9% extracellular hydrocarbons as a percentage of biomass. Showa had the highest hydrocarbon productivity in that study. Those numbers were not obtained under dark fermentation.

Solazyme ultimately commercialized heterotrophic triglyceride oils in other algal chassis rather than commodity Botryococcus hydrocarbons. TerraVia, formerly Solazyme, entered bankruptcy in 2017 and sold assets to Corbion. That history leaves a narrow but worthwhile technical question that can now be tested directly.

Central research question

When the reported heterotrophic method is applied to currently available high-hydrocarbon Botryococcus strains, what recoverable hydrocarbon productivity - measured in grams per liter of reactor volume per day - can actually be achieved, and can inexpensive carbon sources support it?

1. Background

Most algal biofuel programs grow lipid-rich biomass and then convert intracellular triacylglycerols into fuels. B. braunii is different: it synthesizes hydrocarbon molecules directly. Chemical race matters. Race A strains produce odd-numbered C23-C33 alkadienes and alkatrienes; race B strains produce C30-C37 botryococcenes and methylated squalenes; race L produces lycopadiene. Showa and Bot22 are race B strains. UTEX 572, also

circulated as UTEX LB572 / CCAP 807/1 / SAG 807-1, is race A. A method that works well with one race cannot simply be assumed to work with another.

Hydrocarbon content commonly ranges from the mid-teens to about 50% of dry weight, depending on strain and conditions. Older reports of natural B-race populations reaching roughly 75-86% should not be used as fermenter design values. The practical bottleneck remains the amount of hydrocarbon produced per unit volume and time.

2. What Solazyme Actually Reported

The Solazyme patents challenged the assumption that useful *B. braunii* cultivation required photosynthesis. UTEX 572, UTEX 2441, and N-836 (NIES-836) were evaluated on fixed carbon in darkness. Usable sugars were strain-dependent and included glucose, mannose, galactose, and fructose. Dark BG-11 without organic carbon did not support growth.

That finding changes the engineering possibilities. Instead of being limited to illuminated ponds or photobioreactors, at least some strains may be handled in controlled vessels supplied with renewable or waste carbon. Temperature, pH, oxygen, nutrients, and density can then be controlled directly. Whether that translates into better hydrocarbon titer or productivity is the question to be measured, not assumed.

2.1 Experimental observations in the patent specification

- After four weeks in 20 mL BG-11 plus 1% glucose, UTEX 572 with 10 mM urea showed about a 76% increase in growth relative to glucose-only.
- UTEX 2441 with 2 g/L hydrolyzed casein showed up to about a 340% increase.
- N-836 / NIES-836 with 2 g/L hydrolyzed casein showed about a 61% increase.
- A separate UTEX 572 microplate screen visually scored soy flour (~2x), malt extract (~2x), cornstarch (~2.5x), and cornmeal (~3x) above a glucose-only control after 16 days in darkness.

The microplate figures are useful directional evidence for complex agricultural substrates, but they were based on visual well scoring rather than the dry-cell-weight protocol used for the urea and casein comparisons. In a separate flask expansion with 3% glucose, UTEX 572 reached about 9 g/L dry-cell weight in 350 mL in a Fernbach flask, while a parallel 500 mL culture reached only about 3 g/L. That result is best treated as a small-vessel biomass observation, not as a controlled fermenter-scale hydrocarbon productivity.

2.2 Heterotrophy remains strain-dependent

The published record is not uniform. Weetall (1985) reported no heterotrophic dark growth in Texas Culture Collection *B. braunii*, although organic carbon helped in the light. Tanoi et al. (2011) later reported dark growth of strain B70 on glucose and mannose. Wan et al. (2019) grew NIES-836 heterotrophically as seed culture to about 37 g/L in a 5 L fermenter, then transferred those seeds to photoautotrophic hydrocarbon production. That is encouraging evidence for dense seed production, but it is not yet a complete dark hydrocarbon process.

3. Subsequent High-Hydrocarbon Strains

Kleinert and Griehl (Journal of Applied Phycology, 2021) compared twelve strains for non-destructive extraction, or “milking.” Bot22 (race B) contained $51.6 \pm 1.9\%$ extracellular hydrocarbons as a percentage of biomass; Showa (race B), $49.3 \pm 1.4\%$; and CCAP 807/2 (race A), $39.9 \pm 1.2\%$. Showa ranked highest for hydrocarbon productivity and received the top milking score in that study.

These results came from illuminated cultures. They cannot simply be combined mathematically with Solazyme's heterotrophic biomass densities; forcing growth can reduce hydrocarbon fraction. The proposed experiment exists precisely because the combined performance has not been established.

Separate work shows that strain improvement is possible. An EMS mutant of indigenous *B. braunii* AARL G036, designated E1.0H15, was reported at about 1.45x wild-type hydrocarbon productivity. Under optimized

two-stage photosynthetic stress culture, hydrocarbon content reached about 71.6%, with the phenotype reported stable over 24 months (Renewable Energy, 2022). That is not a heterotrophic result, but it demonstrates room for biological improvement.

Faster wild B-race isolates also deserve attention. One screening study reported strain OIT-678 with a doubling time of about 1.2 days and higher biomass and hydrocarbon productivity than Showa under the same photoautotrophic conditions.

4. Research Hypothesis

A high-hydrocarbon strain, grown heterotrophically or under optimized mixotrophy with inexpensive carbon and modern process control, may produce more recoverable hydrocarbon per unit reactor volume and time than historical photosynthetic *Botryococcus* culture.

The primary decision metric will therefore be $\text{g recoverable hydrocarbon} \cdot \text{L}^{-1} \cdot \text{day}^{-1}$. Hydrocarbon percentage, biomass concentration, growth rate, carbon consumption, and extracellular partition are supporting measurements. The project succeeds only if the combined productivity is worth pursuing.

5. Work Plan

Phase I - Reproduce the historical result

Start with a public historical strain, preferably UTEX 572. Parallel phototrophic and dark heterotrophic cultures should use the same inoculum lots. Measure biomass concentration, specific growth rate, residual carbon, hydrocarbon concentration, hydrocarbon class (alkadiene versus botryococcene), and culture axenicity.

Decision point: Continue only if at least one fixed-carbon condition produces a statistically supported dark biomass increase with hydrocarbon identity confirmed. Failure to grow, a collapse of hydrocarbon content, or irreproducible contamination should trigger redesign before more expensive strains are added.

Phase II - Screen high-hydrocarbon strains

Apply the Phase I framework to Showa, Bot22, CCAP 807/2, and NIES-836, subject to availability, deposit terms, and material-transfer agreements. If accessible, include E1.0H15 or an equivalent improved isolate and at least one recently reported fast-growth B-race strain. Rank strains by measured recoverable hydrocarbon productivity, not by hydrocarbon percentage alone.

Decision point: Advance if at least one strain produces recoverable hydrocarbons under heterotrophy or controlled mixotrophy at a volumetric rate that justifies feedstock and recovery optimization.

Phase III - Feedstock economics

Reagent-grade glucose is not a commodity-fuel feedstock. The patent results point toward agricultural and food-processing streams: starch hydrolysates, cornmeal or cornstarch preparations, soy-derived nitrogen, and other low-value carbohydrate streams. Measure yield on carbon - grams of hydrocarbon per gram of substrate consumed - along with growth. A preliminary techno-economic analysis should begin only after Phase II provides measured productivities.

Phase IV - Non-destructive hydrocarbon recovery

Because some strains hold a large share of their hydrocarbons in the extracellular colony matrix, a semicontinuous process may be possible: feed, grow, recover hydrocarbons, and retain the living culture. Published milking work has mainly used illuminated cultures with biocompatible solvents and modest volumetric rates, generally in the milligram-per-liter-per-day range in the cited systems. Recovery efficiency, solvent effects, culture health, and multi-cycle productivity should therefore be measured in the same trophic mode selected for production.

Decision point: Continue only if repeated recovery can be demonstrated without progressive loss of culture viability or productivity.

Phase V - Genetic optimization, if warranted

Genetic engineering should follow, not precede, the strain and trophic-mode screen. The Solazyme patents describe possible modifications of carbon-utilization and hydrocarbon pathways, while other algal patent work describes strategies intended to increase lipid accumulation without the usual starvation growth penalty. Those tools become relevant after the best natural production strain has been identified. Transformation of *B. braunii* remains non-trivial.

6. Intellectual Property and Freedom to Operate

US 8,278,090 B1 was granted 2 October 2012 to Solazyme. A fourth-year maintenance fee was paid; secondary records treat the patent as lapsed for nonpayment after the 7.5-year window. Divisional US 8,956,852 B2, granted 17 February 2015, lapsed on 27 March 2023 for failure to pay the 8-year maintenance fee. Ownership passed from Solazyme to TerraVia Holdings and then Corbion Biotech.

Before commercialization, counsel should review related patent families, extraction and upgrading claims, genetically modified algae, strain deposits, and material-transfer restrictions. Showa, for example, has its own culture and patent history, including ATCC PTA-7441 material. For the research phase, however, the expired specifications provide a useful published experimental starting point.

7. Why Revisit This Now?

The reason to revisit this work is not nostalgia for the algae-fuel programs of fifteen years ago. It is that several pieces now sit next to one another that were developed at different times: a government-supported disclosure of dark *Botryococcus* cultivation; public collections holding relevant strains; later identification of strains approaching 40-50% extracellular hydrocarbons under photoautotrophy; experimental evidence that milking is possible; and better fermentation control and analytical methods.

No published fermenter campaign located in this review appears to have put all of those pieces together and measured the result on the same basis. A limited proof-of-concept study can answer whether doing so is worth further investment.

8. Proposed Deliverables

1. Can the historical Solazyme heterotrophic-growth result be independently reproduced on UTEX 572 or an equivalent public strain?
2. Can high-hydrocarbon strains - especially Showa, Bot22, CCAP 807/2, and NIES-836 - grow under heterotrophic or optimized mixotrophic conditions without losing most of their hydrocarbon content?
3. Which strain produces the greatest recoverable hydrocarbon per liter per day in that trophic mode?
4. Can inexpensive renewable or waste carbon replace laboratory glucose at an acceptable yield?
5. Can non-destructive recovery support repeated hydrocarbon harvests from the same culture?

If those measurements justify further work, the next deliverable is a preliminary techno-economic analysis using measured feedstock cost -> measured reactor productivity -> recovery cost -> hydrocarbon yield -> approximate production cost per gallon-equivalent. Its purpose is to decide whether pilot-scale development is warranted.

9. Ultimate Objective

Determine whether a scalable biological process can convert inexpensive renewable carbon into petroleum-compatible hydrocarbons at a cost that could compete with conventional fuel, without requiring large areas of productive land or continuous illumination.

This is an experimental question. The immediate proposal is simply to answer it in stages, beginning with the least expensive reproducibility test and allowing the measured results to determine whether the project

advances.

Selected Evidence Base

Finding	Evidence and limits
Historical dark cultivation	US 8,278,090 B1 and US 8,956,852 B2; NIST 70NANB7H7002; UTEX 572, UTEX 2441, N-836. Biomass growth in darkness on fixed carbon. Not a peer-reviewed fermenter hydrocarbon campaign.
Nutrient effects	Urea and hydrolyzed casein effects from DCW-style comparisons. Soy/malt/cornstarch/cornmeal ~2-3x on UTEX 572 from visual plate scoring. Flask density ~9 g/L in 350 mL and ~3 g/L in 500 mL.
High-HC strains	Kleinert and Griehl, J. Appl. Phycol. 2021: Bot22 $51.6 \pm 1.9\%$, Showa $49.3 \pm 1.4\%$, CCAP 807/2 $39.9 \pm 1.2\%$ extracellular HC under photoautotrophic milking conditions. Follow-up milking: Showa ~17 and Bot22 ~15 mg HC L ⁻¹ day ⁻¹ .
Strain improvement	E1.0H15 mutant of AARL G036: ~1.45x WT hydrocarbon productivity; ~71.6% HC under two-stage photosynthetic stress; reported stable 24 months (Renewable Energy 2022). Not a heterotrophic result.
Heterotrophy context	Weetall 1985: no dark growth of Texas-collection strains. Tanoi et al. 2011: B70 dark growth on glucose/mannose. Wan et al. 2019: NIES-836 heterotrophic seed to ~37 g/L, then photoautotrophic HC production.
Commercial metric	Primary metric: grams recoverable hydrocarbon per liter reactor volume per day. Do not combine incompatible photoautotrophic HC percentages with heterotrophic biomass titers.
Company outcome	Solazyme/TerraVia commercialized heterotrophic triglyceride oils in other chassis; filed Chapter 11 in 2017; assets acquired by Corbion. Botryococcus fuel was not the surviving product.

Independent verification required. Patent status, freedom to operate, strain-access agreements, deposit terms, and current commercial rights should be verified by qualified counsel before commercialization. This document is a research feasibility framework, not an offer, cost claim, or freedom-to-operate opinion.