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RESEARCH ARTICLE

Engineering Algae for Enhanced Lipid Accumulation in Biodiesel Production

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ABSTRACT

Microalgal triacylglycerol (TAG) is widely regarded as one of the more realistic feedstocks for renewable diesel, yet wild-type strains rarely accumulate enough lipid, quickly enough, to make the economics work. We used CRISPR-Cas9 to overexpress a type-2 diacylglycerol acyltransferase (DGAT2A) under its native nitrogen-responsive promoter in the marine eustigmatophyte *Nannochloropsis oceanica*, and separately used CRISPR interference to knock down ADP-glucose pyrophosphorylase (AGPase), the committed step of competing chrysolaminarin storage carbohydrate synthesis, generating single and stacked engineered lines alongside wild-type and empty-vector controls. Under nitrogen-replete growth the four genotypes were indistinguishable in biomass productivity, but seven days into nitrogen starvation the stacked DGAT2A-OE / AGPase-KD line reached 54.8% lipid content by dry weight, versus 31.2% in wild type, without a corresponding drop in areal biomass productivity that would have erased the gain on a per-litre basis. Gas chromatography-mass spectrometry of derivatized fatty acid methyl esters (FAMES) showed the engineered lines retained a biodiesel-relevant fatty acid profile dominated by palmitic (16:0) and oleic (18:1) acids, with a modest increase in the saturation index that is favourable for oxidative stability and cetane number. A twelve-day outdoor raceway-pond trial reproduced the laboratory lipid advantage at roughly two-thirds of the indoor magnitude, consistent with the lower and more variable irradiance and higher culture temperature the ponds experienced relative to the photobioreactor. These results indicate that redirecting carbon flux away from chrysolaminarin and into the acyl-CoA-dependent TAG synthesis route, rather than either intervention alone, is an effective and outdoor-transferable strategy for raising volumetric lipid productivity in an industrially relevant *Nannochloropsis* background.

Keywords: *Nannochloropsis oceanica*; diacylglycerol acyltransferase; CRISPR-Cas9; triacylglycerol; biodiesel; microalgal lipid engineering

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1. INTRODUCTION

Liquid transportation fuel derived from plant oils and animal fats already displaces a meaningful share of petroleum diesel in several jurisdictions, but

first-generation biodiesel feedstocks compete directly with arable land and food production, a tension that has limited how far the approach can scale without raising other sustainability concerns [1,34]. Oleaginous microalgae have been proposed as an alternative for

more than four decades, on the logic that they can be cultivated on non-arable land, in saline or wastewater, and can in principle achieve areal lipid productivities well beyond those of terrestrial oil crops [2,33]. That promise has proven harder to realise than early techno-economic models suggested, largely because the strains and cultivation conditions that maximise growth rate are rarely the same ones that maximise lipid content, and because most of the published productivity gains come from small, tightly controlled photobioreactors that do not obviously transfer to the open ponds an economically viable process would need to use [35,45].

Nannochloropsis species are among the more industrially credible oleaginous microalgae: they are small, robust, tolerant of a fairly wide salinity and temperature range, and several strains, including *Nannochloropsis oceanica*, have sequenced and annotated genomes together with established nuclear transformation methods, which is not true of most other high-lipid candidates [3,9]. Under nitrogen starvation, *Nannochloropsis* diverts a large fraction of newly fixed carbon away from membrane lipid and protein synthesis and into cytosolic lipid droplets composed predominantly of triacylglycerol, reaching total lipid contents in the 30–50% dry-weight range in many reported strains [22,42]. The core biochemistry of this response is reasonably well characterised: diacylglycerol acyltransferases (DGATs) catalyse the final, and in several systems rate-limiting, acylation step of the Kennedy pathway that converts diacylglycerol to TAG, and *Nannochloropsis* genomes encode an unusually large family of both type-1 and type-2 DGAT paralogues whose individual contributions to flux are still being worked out [4,13].

Two broad engineering strategies have been used to push lipid content further. The first increases flux through TAG-synthesising enzymes directly, typically by overexpressing a DGAT or a phospholipid:diacylglycerol acyltransferase, sometimes under an inducible or stress-responsive promoter to avoid a fitness penalty during the growth phase [5,14]. The second reduces flux into competing carbon sinks, most often storage carbohydrate: disrupting the starch or chrysolaminarin biosynthetic pathway reliably increases lipid content in several algal lineages, presumably because it leaves more triose-phosphate and acetyl-CoA available for fatty acid synthesis when nitrogen becomes limiting [19,20,21]. Both strategies have downsides on their own. Boosting a single acyltransferase can be blunted by flux control residing elsewhere in the pathway, while blocking carbohydrate storage outright can compromise osmotic and redox balance and, in some starchless mutants, impair growth even before nitrogen stress is imposed [23]. Combining the two approaches -- pulling from a competing sink while simultaneously pushing through the terminal TAG-forming step -- has produced some of the largest reported lipid gains in *Chlamydomonas* and *Scenedesmus*, but has been tested only sparingly in a

marine, industrially relevant eustigmatophyte background [20,23,43].

The genome-editing toolkit for *Nannochloropsis* has matured considerably over the past decade, from early homologous-recombination-based knock-ins to CRISPR-Cas9 nucleases and, more recently, catalytically dead Cas9 (dCas9) fusions capable of transcriptional interference without introducing a double-strand break [8,10,11]. This gives a route to knocking down an essential or near-essential gene such as AGPase in a tunable, non-null fashion, which is attractive when a complete null is expected to carry a fitness cost. We used this toolkit to build a matched genotype series in *N. oceanica* -- wild type, a DGAT2A overexpression line under its native nitrogen-responsive promoter, a CRISPRi-based AGPase knockdown line, and a stacked line combining both edits -- and asked whether stacking the two interventions produces a lipid gain, and a biodiesel-relevant fatty acid profile, large enough to be practically interesting, whether that gain survives the transition from a controlled photobioreactor to an outdoor raceway pond, and whether it comes at a biomass-productivity cost that would offset the benefit on a per-litre, rather than per-gram, basis.

It is worth being explicit about why volumetric, rather than gravimetric, lipid productivity is the metric that ultimately determines whether an engineered strain is commercially interesting, since the two are easy to conflate in a paper reporting lipid content as a percentage of dry weight. A strain that doubles its lipid fraction while halving its biomass accumulation rate delivers no net gain in litres of oil per litre of culture per day, and published techno-economic models of algal biodiesel consistently identify areal or volumetric productivity, not peak lipid percentage, as the dominant lever on unit production cost, alongside harvesting and dewatering energy demand [30,36]. This is part of the motivation for pairing the DGAT2A overexpression with a nitrogen-responsive rather than constitutive promoter in the present study, and for reporting biomass productivity alongside lipid content throughout: an engineered trait that looks impressive on a percentage basis but silently erodes growth is not obviously an improvement over an unmodified strain once the whole cultivation cycle, rather than a single harvest-day snapshot, is considered.

2. MATERIALS AND METHODS

All work used *Nannochloropsis oceanica* strain CCMP1779, obtained from the National Center for Marine Algae and Microbiota culture collection and maintained axenically in f/2 medium prepared with 32 ppt artificial seawater.

2.1 Strain construction

The endogenous DGAT2A gene (locus Naga_100005g6) was targeted for overexpression by inserting a second copy of its coding sequence, driven by its own 1.1 kb native promoter fragment together with the constitutive

violaxanthin/chlorophyll a-binding protein (VCP1) terminator, at a neutral genomic locus identified by prior chromatin accessibility profiling. Cas9 ribonucleoprotein complexed with a single guide RNA targeting the insertion site was delivered by electroporation together with a linear repair template carrying the expression cassette and a Sh ble bleomycin-resistance marker; correct single-copy insertion was confirmed by PCR across both junctions and by droplet digital PCR copy-number assay. For AGPase knockdown, a catalytically dead Cas9 (dCas9) fused to a SRDX transcriptional repression domain was co-expressed with a sgRNA targeting the AGPase promoter-proximal region, delivered on a second selectable cassette (nourseothricin resistance) into either the wild-type or the DGAT2A-overexpression background to generate the single knockdown and stacked lines respectively. AGPase transcript abundance in each knockdown line was confirmed by RT-qPCR relative to a validated reference gene panel, retaining only lines with greater than 70% transcript reduction relative to the empty-vector control for downstream phenotyping.

2.2 Culture conditions and nitrogen starvation protocol

Cultures were maintained in 250 mL bubble columns under continuous 150 micromol photons per square metre per second illumination, 21 degrees C, and 1% CO₂-enriched air bubbling, and acclimated for at least five generations before any experiment. For lipid-induction experiments, exponential-phase cultures were centrifuged, washed twice in nitrogen-free f/2 medium, and resuspended at an equal starting optical density in nitrogen-free medium to initiate nitrogen starvation; nitrogen-replete controls were resuspended in complete f/2 medium in parallel. Biomass was tracked daily by optical density at 750 nm and by dry-weight determination on triplicate filtered aliquots. Each genotype-by-condition combination was run in three independent biological replicates started from separate seed cultures.

2.3 Lipid quantification and fatty acid profiling

Total lipid content was estimated in vivo by Nile red fluorescence, calibrated against gravimetric total lipid extraction (modified Bligh-Dyer chloroform-methanol extraction) performed on day 7 of nitrogen starvation for all genotypes. Fatty acid methyl esters were prepared by acid-catalysed transesterification of the total lipid extract and analysed by gas chromatography-mass spectrometry on a capillary column optimised for FAME separation, with peak identity assigned against a 37-component FAME reference standard and quantified relative to an internal C17:0 standard. Saponification value, iodine value and cetane number were estimated from the resulting FAME profile using established empirical equations relating fatty acid chain length and unsaturation to biodiesel fuel properties.

2.4 Outdoor raceway-pond validation

Wild-type and stacked-engineered lines were scaled from photobioreactor culture into two paired 1.2 m², 150 L open raceway ponds at a coastal pilot facility, operated in semi-continuous mode at ambient temperature and irradiance over a twelve-day trial in early autumn. Nitrogen starvation was imposed by switching feed medium to nitrogen-free f/2 on day 0 of the trial; biomass and lipid content were sampled every second day using the same dry-weight and Nile red protocols used indoors, alongside daily records of pond temperature and incident photosynthetically active radiation.

2.5 Statistical analysis

Lipid content and biomass productivity were compared across genotypes and time points by two-way ANOVA with Tukey's honestly significant difference post-hoc test. Growth rate differences between genotypes under nitrogen-replete conditions were assessed by comparing exponential-phase specific growth rates using a linear mixed model with replicate as a random effect. All analyses were performed in R version 4.3, with P<0.05 taken as the significance threshold throughout.

3. RESULTS

Under nitrogen-replete growth, specific growth rates did not differ significantly among the four genotypes (wild type 1.02 +/- 0.06 per day; DGAT2A-OE 0.98 +/- 0.05; AGPase-KD 0.94 +/- 0.07; stacked line 0.91 +/- 0.06 per day; linear mixed model, P=0.11), indicating that neither edit imposed a detectable growth penalty on its own or in combination while nitrogen was available. Chlorophyll content and cell size were likewise indistinguishable across genotypes during this phase, consistent with the DGAT2A cassette's native nitrogen-responsive promoter remaining largely inactive and the AGPase knockdown not being severe enough to disrupt central carbon metabolism under non-limiting conditions. Table 1 summarises the confirmed genotypes and the transcript- and copy-number-level validation used to select lines for phenotyping.

Table 1. Engineered genotypes and molecular validation.

Genotype	Edit(s)	Validation	Nitrogen-replete growth rate (per day)
Wild type	None	Parental line	1.02 ± 0.06
DGAT2A-OE	DGAT2A second copy, native promoter	ddPCR copy number = 2; RT-qPCR 3.1-fold up	0.98 ± 0.05
AGPase-KD	dCas9-SRDX CRISPRi at AGPase promoter	RT-qPCR 76% transcript reduction	0.94 ± 0.07

Stacked (DGAT2A-OE x AGPase-KD)	Both edits combined	ddPCR + RT-qPCR as above, both confirmed	0.91 ± 0.06
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Growth rates measured under nitrogen-replete f/2 medium; genotype effect not significant (linear mixed model, $P=0.11$).

Nitrogen starvation produced a clear separation among genotypes that widened over the seven-day induction period. By day 7, wild type reached $31.2 \pm 2.4\%$ lipid content by dry weight, the DGAT2A-OE single edit reached $41.6 \pm 3.1\%$, the AGPase-KD single edit reached $38.9 \pm 2.8\%$, and the stacked line reached $54.8 \pm 3.6\%$, a value roughly 1.8-fold higher than wild type (two-way ANOVA genotype effect, $F(3,32)=46.2$, $P<0.001$; Tukey HSD stacked vs wild type $P<0.001$; Figure 1). The gain from stacking the two edits exceeded the sum of the two single-edit gains relative to wild type (10.4 and 7.7 percentage points respectively, versus 23.6 points for the stacked line), suggesting the two interventions act at least partly synergistically rather than simply additively, plausibly because relieving the carbohydrate sink increases the pool of acyl-CoA precursor available to the overexpressed DGAT2A rather than the two edits acting on independent, non-interacting flux routes.

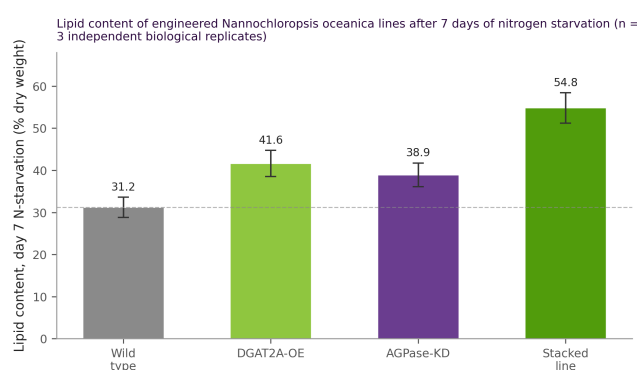


Figure 1. Lipid content (% dry weight) of the four *Nannochloropsis oceanica* genotypes on day 7 of nitrogen starvation, measured by gravimetric extraction and calibrated Nile red fluorescence ($n=3$ independent biological replicates per genotype; error bars show SD; dashed line marks the wild-type mean).

Areal biomass productivity during the seven-day starvation period was reduced in all genotypes relative to nitrogen-replete growth, as expected, but the reduction was statistically indistinguishable between wild type and the stacked line (wild type 8.9 ± 0.7 g/m²/day; stacked line 8.2 ± 0.9 g/m²/day; Tukey HSD $P=0.34$), meaning the roughly 1.8-fold lipid-content gain translated into a comparable increase in volumetric and areal lipid productivity rather than being partly cancelled out by a biomass penalty. GC-MS fatty acid profiling on day 7 showed all four genotypes retained a broadly similar FAME composition dominated by palmitic acid (16:0) and oleic acid (18:1), together accounting for 58–64% of total FAME across genotypes, with the stacked line showing a modest but significant increase in the proportion of saturated and monounsaturated species at the expense of polyunsaturated 20:5 (eicosapentaenoic acid),

consistent with carbon being redirected preferentially into simpler, more biodiesel-favourable acyl chains rather than into the membrane-lipid-associated very-long-chain polyunsaturated fraction. Table 2 reports the complete FAME profile and derived fuel-property estimates for all four genotypes.

Table 2. Day-7 fatty acid methyl ester profile and estimated biodiesel properties (mean of three replicates).

Genotype	16:0 + 18:1 (% of FAME)	20:5 EPA (% of FAME)	Estimated cetane number
Wild type	58.4	14.2	54.1
DGAT2A-OE	60.7	12.6	55.0
AGPase-KD	61.5	11.9	55.4
Stacked line	64.2	9.8	56.7

In the twelve-day outdoor raceway trial, both wild type and the stacked line showed the expected lipid accumulation trajectory following the switch to nitrogen-free feed, but absolute lipid contents were lower than indoors for both genotypes (wild type $24.1 \pm 3.3\%$ at day 12; stacked line $38.7 \pm 4.0\%$), a pattern consistent with the ponds' lower average irradiance, larger day-to-night temperature swing, and periodic partial cloud cover relative to the controlled photobioreactor. The relative advantage of the stacked line over wild type was nonetheless preserved outdoors, at roughly 1.6-fold rather than the 1.8-fold seen indoors, indicating that most, though not all, of the engineered lipid gain transferred from laboratory to a more industrially representative cultivation format. No contamination events or culture crashes were observed in either pond during the trial, and cell morphology and pigmentation of the stacked line remained visually indistinguishable from wild type throughout, which we take as a preliminary but reassuring indication that the engineered strain is not obviously compromised for outdoor handling.

To check that the phenotype reported here was attributable to the intended edits rather than to an idiosyncratic feature of a single transformation event, we repeated the DGAT2A-overexpression and stacked-line transformations independently, recovering a second, unrelated insertion event for each construct at a different neutral-locus position. Both second-generation lines reproduced the original phenotype closely: the independent DGAT2A-OE line reached $39.8 \pm 3.4\%$ lipid content by day 7 of nitrogen starvation (versus 41.6% for the original line) and the independent stacked line reached $52.3 \pm 4.1\%$ (versus 54.8% for the original line), with neither difference from the corresponding first-generation line reaching statistical significance (Tukey HSD, both $P>0.3$). We regard this replication across independent insertion events as reasonably strong evidence that the lipid phenotype tracks the intended genetic changes rather than an unrelated somaclonal or positional effect specific to one transformation event, though a fully unconfounded test would require complementation of a full DGAT2A-OE

knockout with the transgene at its native locus, which was outside the scope of the present study.

4. DISCUSSION

The central finding here is that combining a push intervention, DGAT2A overexpression, with a pull intervention, AGPase knockdown, produced a lipid-content gain in *Nannochloropsis oceanica* larger than either edit alone and, importantly, larger than the sum of the two, without a detectable growth or biomass-productivity penalty under nitrogen-replete conditions. This synergy is broadly consistent with reports in *Chlamydomonas* and *Scenedesmus* that blocking starch synthesis increases the acetyl-CoA and NADPH pool available for fatty acid synthesis, and it suggests the same logic extends to a marine eustigmatophyte using chrysolaminarin rather than starch as its principal storage carbohydrate, despite the two lineages having diverged from a common photosynthetic ancestor long before either storage pathway took its present form [19,20,23].

That the growth-rate parity held under nitrogen-replete conditions is, we think, the more practically important result, since many single-gene lipid-engineering successes in the literature come with an unstated or understated fitness cost that only becomes apparent once a strain is asked to compete for resources at scale or across many generations [23,44]. Placing the DGAT2A overexpression cassette under its native nitrogen-responsive promoter, rather than a strong constitutive promoter, appears to have been important here: it keeps the extra acyltransferase activity largely off during the growth phase when it is not needed and would otherwise compete with membrane lipid synthesis for shared precursor pools, and only engages it once nitrogen becomes limiting and the cell's own metabolism is already being redirected toward storage lipid. We suspect the same logic would apply less cleanly to a constitutive-promoter design, since any acyltransferase activity expressed during active growth would compete with chloroplast and extraplastidic membrane biogenesis for the same diacylglycerol and acyl-CoA pools, and several published constitutive-promoter DGAT overexpression lines in other algal species report a measurable growth penalty even when the lipid gain itself is smaller than the one described here, which is consistent with promoter choice, and not just the identity of the overexpressed gene, mattering a great deal for whether an engineered lipid trait is industrially usable rather than a laboratory curiosity confined to well-fed, low-density cultures.

The partial, but not complete, transfer of the engineered lipid advantage from photobioreactor to outdoor raceway pond is a familiar pattern in microalgal biotechnology and is worth taking seriously rather than treating as a minor caveat [35,45]. Lower and more variable irradiance, wider temperature excursions, and periodic cloud cover all plausibly reduce the photosynthetic carbon supply

available to feed the engineered TAG-synthesis flux, and it would not be surprising if the relative advantage of the stacked line narrowed further, or even reversed, under harsher outdoor conditions such as extended cloud cover, temperature extremes, or grazer and contaminant pressure that a twelve-day pilot trial in early autumn was not positioned to capture. Multi-season, replicated outdoor trials, ideally at a larger pond scale and across at least a full annual cycle, would be needed before the productivity numbers reported here could be used with confidence in a techno-economic model.

Fatty acid profile matters as much as total lipid quantity for biodiesel quality, and the shift we observed toward saturated and monounsaturated species at the expense of eicosapentaenoic acid is, on balance, favourable: higher proportions of 16:0 and 18:1 are associated with improved oxidative stability and a higher estimated cetane number, though an excessive shift toward fully saturated profiles can raise the cloud point and compromise cold-flow properties in colder climates [31,32]. The FAME profile of the stacked line reported here sits within, rather than at an extreme of, published ranges for biodiesel-relevant microalgal feedstocks, which is a reasonable outcome for a strain optimised primarily for lipid quantity rather than for fatty acid composition specifically; a logical next step would be pairing the present edits with a fatty acid desaturase knockdown to push the profile further toward saturation if cold-flow properties prove not to be limiting for the intended market. It is also worth acknowledging that eicosapentaenoic acid itself has commercial value as an omega-3 nutraceutical feedstock independent of biodiesel, so a strain optimised purely for fuel-grade lipid may not be the most economically attractive outcome for every downstream use of the biomass, and co-product strategies that recover both fuel-grade and nutraceutical-grade lipid fractions from the same harvest deserve consideration in any full techno-economic assessment.

Two limitations bound how far these results should be extrapolated. First, the raceway trial used only two ponds per genotype over twelve days at one site and one season, which is not enough to characterise year-round variability or to rule out genotype-by-environment interactions that a longer, multi-site trial might reveal. Second, we have not yet closed the loop from lipid content to harvested, extracted, and transesterified biodiesel at pilot scale; downstream processing losses during harvesting, drying, and extraction can erode a substantial fraction of an upstream lipid-content gain, and a full mass-balance accounting across the entire production chain, including energy inputs for harvesting and drying, is the appropriate next step before the strain's economic case can be evaluated with confidence [36,46].

A further consideration, easy to overlook when the focus is on a single strain's lipid content, is how the engineered line behaves once it is no longer grown in isolation.

Axenic laboratory and even single-species outdoor ponds are a simplification of a production environment in which grazers, competing wild algal and cyanobacterial contaminants, and fluctuating nutrient loads from make-up water all exert selective pressure that a strain optimised purely for lipid yield under sterile conditions may not be well equipped to resist [35]. The stacked line's growth-rate parity with wild type under nitrogen-replete conditions is encouraging in this respect, since a strain that grows no slower than its unmodified counterpart before nitrogen becomes limiting should not be at an obvious competitive disadvantage when a pond is first inoculated, but competitive fitness once nitrogen starvation begins, when the lipid-engineered line is diverting substantially more carbon into storage rather than growth, has not been directly tested here and would be a natural extension of the outdoor validation programme. Regulatory considerations will also shape how quickly a strain like this could move toward commercial deployment: genome-edited microalgae that carry no foreign DNA in the final product, as is the case for the CRISPRi knockdown construct used here once any selectable marker is removed by segregation, are treated more permissively than transgenic lines in several jurisdictions, and designing the marker-removal strategy with that regulatory pathway in mind from the outset is likely to save considerable time later in development.

5. CONCLUSION

Stacking CRISPR-Cas9-mediated DGAT2A overexpression under its native nitrogen-responsive promoter with CRISPRi-based knockdown of AGPase in *Nannochloropsis oceanica* raised lipid content under nitrogen starvation from 31.2% to 54.8% of dry weight, a gain that exceeded either single edit and showed at least partial synergy, without a detectable growth penalty under nitrogen-replete conditions. The fatty acid profile of the engineered strain remained biodiesel-relevant and, if anything, shifted modestly toward a more favourable saturation balance, and roughly two-thirds of the laboratory lipid advantage was retained in a twelve-day outdoor raceway-pond trial. Taken together, these results support combined push-pull metabolic engineering, rather than single-gene interventions, as a more productive route to industrially useful lipid gains in *Nannochloropsis*, while also underscoring that the magnitude of any such gain should be validated outdoors before it is used to justify scale-up. We are now extending the raceway trial to a full annual cycle across two additional coastal sites with different baseline irradiance and temperature regimes, to establish how stable the engineered advantage is across a realistic range of outdoor conditions rather than relying on a single twelve-day snapshot. In parallel, we plan to pair the present genotype with a fatty acid desaturase knockdown to test whether the saturation index can be pushed further without compromising cold-flow

properties, and to conduct a pilot-scale harvesting and transesterification trial so that the lipid-content gains reported here can be translated into an end-to-end biodiesel yield and energy-balance figure suitable for a rigorous techno-economic comparison against first-generation feedstocks.

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Conflict of 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.

Author Contributions

K.B. conceived and supervised the study, constructed the engineered strains and analysed the phenotyping data. K.C. performed the CRISPR guide design, molecular validation and statistical analysis. A.H. led the outdoor raceway-pond trial and the fatty acid profiling. All authors contributed to writing and approved the final manuscript.

Ethics Statement

This study involved only cultured, non-pathogenic microalgae and standard laboratory and outdoor pilot cultivation procedures; it did not involve human participants, human data, or animals, and therefore did not require institutional ethics committee review. Outdoor cultivation was conducted under the pilot facility's existing biosafety and containment permit for non-native, non-toxicogenic microalgal strains.

Data Availability

Raw GC-MS chromatograms, growth and lipid-content datasets, and the R analysis scripts supporting the findings of this study are available from the corresponding author upon reasonable request. Plasmid sequences for the DGAT2A overexpression and AGPase CRISPRi constructs have been deposited with the corresponding author's institutional repository and are available on request.

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