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Article

Engineering the Novel Extremophile Alga *Chlamydomonas pacifica* for High Lipid and High Starch Production as a Path to Developing Commercially Relevant Strains

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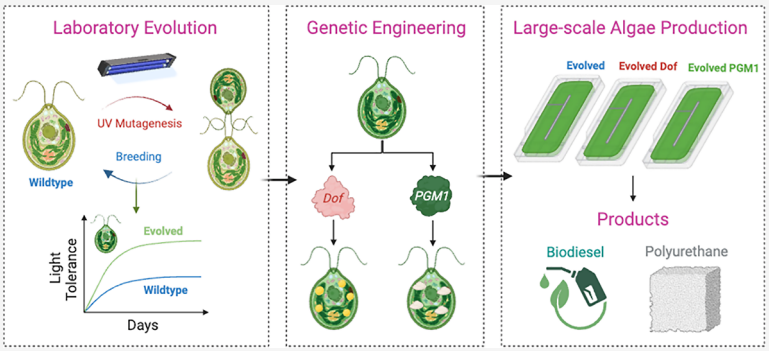

ABSTRACT: Microalgae offer a compelling platform for the production of commodity products, due to their superior photosynthetic efficiency, adaptability to nonarable lands and nonpotable water, and their capacity to produce a versatile array of bioproducts, including biofuels and biomaterials. However, the scalability of microalgae as a bioresource has been hindered by challenges such as costly biomass production related to vulnerability to pond crashes during large-scale cultivation. This study presents a pipeline for the genetic engineering and pilot-scale production of biodiesel and thermoplastic polyurethane precursors in the extremophile species *Chlamydomonas pacifica*. This extremophile microalga exhibits exceptional resilience to high pH (>11.5), high salinity (up to 2% NaCl), and elevated temperatures (up to 42 °C). Initially, we evolved this strain to also have a high tolerance to high light intensity (>2000 $\mu E/m^2/s$) through mutagenesis, breeding, and selection. We subsequently genetically engineered *C. pacifica* to significantly enhance lipid production by 28% and starch accumulation by 27%, all without affecting its growth rate. We demonstrated the scalability of these engineered strains by cultivating them in pilot-scale raceway ponds and converting the resulting biomass into biodiesel and thermoplastic polyurethanes. This study showcases the complete cycle of transforming a newly discovered species into a commercially relevant commodity production strain. This research underscores the potential of extremophile algae, including *C. pacifica*, as a key species for the burgeoning sustainable bioeconomy, offering a viable path forward in mitigating environmental challenges and supporting global bioproduct demands.

KEYWORDS: *sustainability, biotechnology, microalgae, biofuels, biomaterial*

INTRODUCTION

As the 21st century progresses, humanity faces unprecedented challenges in the form of climate change and a rapidly growing global population.[1,2] These twin crises threaten food security, strain conventional energy sources, and exacerbate environmental degradation.[3−5] The quest for sustainable solutions has never been more urgent, compelling the exploration of innovative strategies that can provide the next generation of food and fuel without further harming our planet.[6−8] In this

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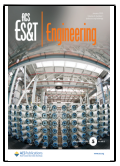

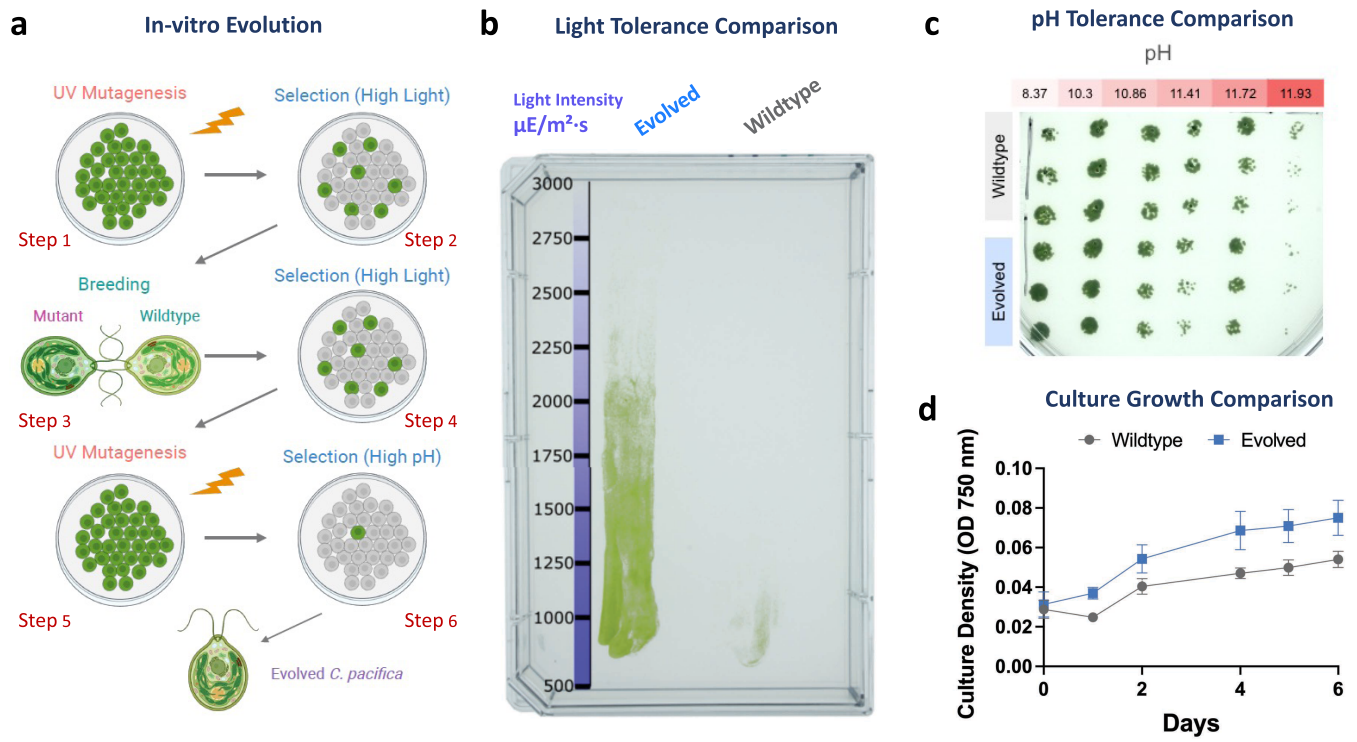

Figure 1. Laboratory evolution of *C. pacifica* for high light tolerance (a) Methodology used for evolving *C. pacifica* to gain high light tolerance, including mutagenesis and breeding (Steps 1−6). (b) Growth comparison of evolved and wildtype *C. pacifica* cells on media plates under a light gradient. (c) Tolerance of evolved and wildtype *C. pacifica* cells under different pH conditions. (d) Culture density of evolved and wildtype *C. pacifica* cells. Panel (a) schematic created with BioRender.com.

context, developing renewable resources that can reduce carbon emissions and offer a viable alternative to traditional agricultural and energy production methods is critical. The need for such solutions is underscored by the increasing scarcity of arable land, the depletion of freshwater resources, and the necessity to reduce greenhouse gas emissions.[9,10] As we navigate this pivotal moment in human history, the identification and cultivation of sustainable, efficient, and versatile sources of food and fuel emerge not just as a scientific endeavor but as a vital component of our collective response to the existential challenges of our time.

Against this backdrop of environmental and societal challenges, microalgae emerged as a promising solution with the potential to revolutionize our approach to sustainable food, fuel, and material production.[11−15] Microalgae, with their exceptional photosynthetic efficiency, can convert sunlight into biomass more effectively than traditional crops, offering a high-yield, renewable resource that grows rapidly and requires minimal inputs.[16] Their cultivation does not compete for arable land, as they can thrive in nonarable environments, including deserts and saline waters, utilizing nonpotable water and even wastewater, thereby reducing the strain on freshwater resources.[17] Furthermore, microalgae are capable of producing a wide range of valuable bioproducts, from biofuels that can reduce our dependence on fossil fuels to high-protein biomass for food and feed, along with bioplastics, pharmaceuticals, and cosmetics.[18−22] This versatility positions microalgae as a potential keystone of a circular bioeconomy, capable of addressing both energy and food security while contributing to carbon mitigation efforts.[23] Harnessing the full capabilities of microalgae as a sustainable resource remains fraught with obstacles. Scaling up production to meet the burgeoning global needs introduces a set of complex technical and financial challenges.[24,25] Chief among these is the necessity to sustain stable cultivation environments on a large scale, a task complicated by the risk of contamination and the variability of environmental factors, which can precipitate culture crashes and disrupt biomass yield.[26,27] For example, although *Chlamydomonas reinhardtii*, a model green alga, thrives in closed bioreactors where growth conditions can be carefully controlled, it is not well-suited for large-scale cultivation in open systems due to the risk of contamination from zooplankton grazers and other unpredictable environmental factors.[26,28−32]

A promising strategy to navigate the hurdles associated with large-scale microalgae cultivation involves focusing on extremophile strains of microalgae.[33−37] These organisms naturally thrive in extreme conditions, such as high salinity, extreme temperatures, acidic or alkaline environments, where conventional microalgae strains would struggle or fail to survive. By leveraging the inherent resilience of these extremophiles, it is possible to reduce the risk of pond crashes due to environmental stressors, thus ensuring a more stable and reliable biomass production. Furthermore, the genetic engineering of these extremophile strains to enhance their cellular productivity opens new avenues for bioproduct production optimization.[38−40] Through targeted modifications, these strains can be engineered to have increased lipid, carbohydrate, or protein content, thereby maximizing the yield of desired bioproducts. This dual approach of utilizing extremophile microalgae and enhancing their productivity through genetic engineering not only mitigates some of the challenges of stable large-scale cultivation but also paves the way for economically viable and environmentally sustainable

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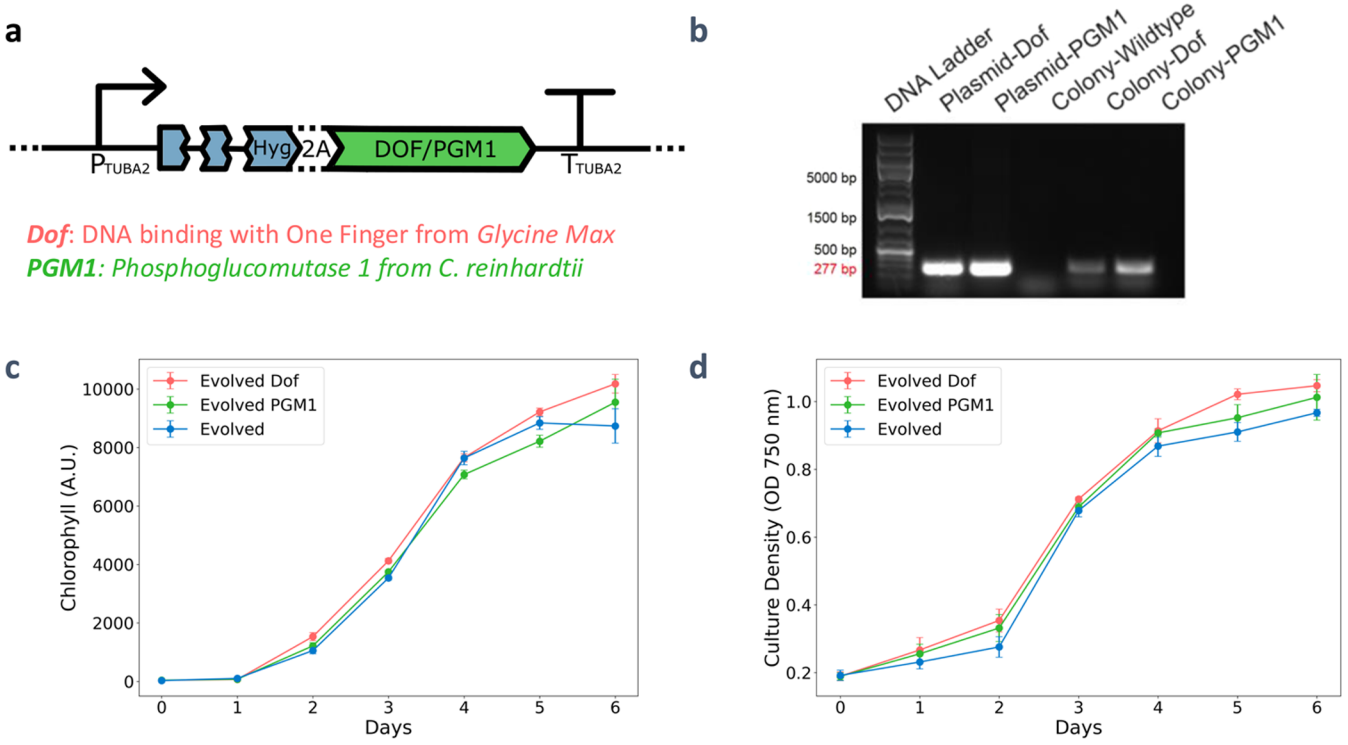

Figure 2. Metabolic engineering of *C. pacifica* for high cellular lipid and starch production (a) Plasmid construct showing β-2-tubulin promoter, target genes, and selectable markers Hygromycin. (b) Colony PCR confirmation of transformants. (c, d) Growth curves showing chlorophyll and culture density of evolved Dof and evolved PGM1 transformants w.r.t. evolved wildtype strain, respectively. F2A represents a self-cleaving sequence. A.U. denotes Arbitrary Unit.

production of biofuels, food, and other valuable bioproducts. This innovative strategy harnesses the robustness of extremophiles against environmental challenges while pushing the boundaries of their natural productivity, offering a holistic solution to the sustainability puzzle posed by the increasing demands for food and fuel in the face of climate change.

In this study, we showcase a complete comprehensive pipeline from the genetic engineering of extremophile microalgae in the laboratory to their pilot-scale production for biodiesel and thermoplastic polyurethane synthesis. Our research centers on the novel extremophile species *Chlamydomonas pacifica* (402 and 403; Chlamydomonas Resource Center ID, CC-5697, and CC-5699, respectively), which our laboratory discovered in San Diego, California.[41] This microalga is particularly suited for industrial applications due to its exceptional resilience to extreme environmental conditions, such as high pH, high salinity, and elevated temperatures, its ability for mating and high throughput selection, and its ability for recombinant gene expression.[41] Initially, we enhanced the strain's tolerance to high light intensity through mutagenesis, breeding, and selection. Following this, we employed genetic engineering techniques to develop *C. pacifica* strains that produce high levels of lipids and starch without compromising their growth rates. We successfully demonstrated the scalability of these engineered strains by cultivating them in pilot-scale raceway ponds and converting the resulting biomass into biodiesel and thermoplastic polyurethane (TPU). This study is pioneering in showcasing the complete cycle of transforming a newly discovered species into a commercially relevant strain. The research highlights the potential of *C. pacifica* as a key microalgae species in the sustainable bioeconomy, offering a viable solution to environmental challenges and supporting global demands for renewable resources.

RESULTS

Enhancing Light Tolerance through In Vitro Evolution. Given the strain's natural resilience to high pH, salinity, and temperature, we aimed to identify if we could enhance its tolerance to high light intensity, as well. This improvement would be crucial because high light is known to have an inhibitory effect on microalgae growth.[42−45] To achieve this, we leveraged mutagenesis and breeding approaches, which have been previously shown to improve traits in microalgae.[46−51]

Briefly, we performed UV mutagenesis on the wild-type *C. pacifica* 402 and selected mutants that thrived under high-light conditions (Steps 1−2; Figure 1a; see Methods section). The selected mutants were mated or subjected to genome shuffling through sexual recombination with a wild-type *C. pacifica* 403 (Step 3). This process preserves genome stability while allowing for genetic variation by eliminating undesirable mutations.[49] Next, the progenies were selected under high light, to identify those strains that showed a stable resistance to high light, to ensure the retainment of the high-light tolerance trait (Step 4; Figure 1a and Supporting Figure 2A). We conducted an additional round of UV mutagenesis and selection to check if we could further enhance the resilience of the strain to commercially relevant high pH conditions (Steps 5−6; Figure 1a and Supporting Figure 2B). The resultant strain, referred to as the evolved strain, showed remarkable tolerance when exposed to light intensities ranging from 500 to 3000 $\mu E/m^2/s$ for 24 h, followed by recovery under low light (80 $\mu E/m^2/s$) (Figure 1b). The evolved strain

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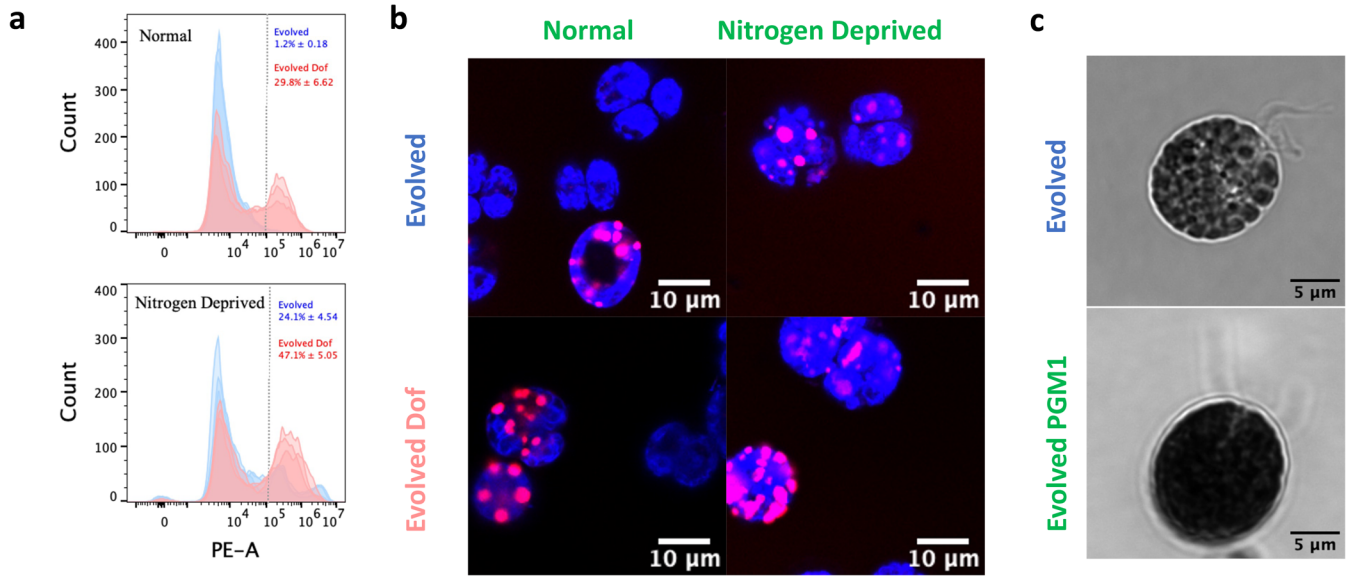

Figure 3. Characterization of evolved *Dof* and evolved PGM1 strains (a) Flow cytometry data of Nile red-stained cells, quantifying lipid content in high lipid-producing *C. pacifica* cells. (b) Confocal microscopy images comparing evolved and evolved *Dof* strains, highlighting lipid accumulation. (c) DIC microscopy images of Evolved PGM1 strain compared to Evolved wildtype stained with Lugol solution under sulfur starvation. The gray dotted line in panel (a) represents a threshold 10^5, and PE-A corresponds to the phycoerythrin-area channel. Panel (a) shows three curves corresponding to three different biological replicates.

survived light intensities above 2000 $\mu E/m^2/s$, whereas the wild-type strain succumbed around 1000 $\mu E/m^2/s$, indicating a 2-fold improvement in light tolerance. Moreover, when grown in liquid media, the evolved strain demonstrated approximately a 38.78% increase in culture density at 750 nm after 6 days of growth under 400 $\mu E/m^2/s$ and pH 10.25 (Figure 1d; p = 0.02; n = 3; two-tailed t test).

Although we conducted mutagenesis and selection in the second round to enhance commercially relevant high pH tolerance, and further tested for this trait, we were unable to observe a definitive difference in pH tolerance between the wild type and the evolved strains (Figure 1c). This may be due to the cells already achieving their maximum pH tolerance, which remains very high (exceeding pH 11.75), or the loss of the phenotype after subculturing due to the presence of other deleterious mutations in the mutant. A following mating step could be used to stabilize it by reconstituting the deleterious mutations and keeping it beneficial, but this was not pursued at the time.[49] We suspect the Evolved strain is of the *C. pacifica* 403 mating type, as coculturing it with *C. pacifica* 402 and 403 separately revealed signs of cell wall release and early stages of mating cluster formation in the cultures involving the Evolved strain and *C. pacifica* 402 (Supporting Figure 4).

Nonetheless, the evolved strain's high light tolerance was assessed over a period exceeding a year, with the phenotype remaining stable throughout (Figure 1). From this point forward, all experiments were conducted using the evolved strain. Overall, these significant improvements highlight the strain's enhanced adaptability to adverse environmental conditions, validating our approach to genetically refining microalgae for superior resilience to outdoor cultivation. Such advancements not only demonstrate the potential of Evolved *C. pacifica* to overcome common cultivation challenges and establish a foundation for advancing large-scale, sustainable microalgae production.

Genetic Engineering of the Evolved Strain for Enhanced Lipid and Starch Production. *High Lipid.* To enhance the cellular output of the evolved *C. pacifica* strain, we adopted a metabolic engineering strategy centered around transcription factors.[39,52] Specifically, we overexpressed a soybean-derived *Dof* (DNA binding with one finger) transcription factor, recognized for its effectiveness in boosting lipid production within various plant and microalgae species, including *Arabidopsis*, *Chlamydomonas reinhardtii*, *Chlorella ellipsoidea*, and *Chlorella vulgaris*.[53−58] This family of transcription factors is notable for its heightened responsiveness and greater efficacy in stimulating lipid production, particularly under conditions of nitrogen depletion. We also attempted to identify any endogenous *Dof* transcription factors in *C. pacifica* using iTAK, TAPscan, and PlantTFDB transcription factor identification tools.[59−61] However, none of these tools identified any transcription factors belonging to the *Dof* family in *C. pacifica*. This could be due to the current genome annotation quality limitations for this new species or the evolutionary loss of this transcription factor in *C. pacifica*. Consequently, we proceeded with the exogenous *Dof* transcription factor sourced from the soybean (*Glycine max*) plant (Figure 2a,b). Using RT-qPCR, we observed that the evolved and genetically modified strain (Evolved *Dof*) exhibited a 106-fold relative abundance (Cq = 33.8 ± 0.53) compared to the wild-type (Cq = 39.4 ± 1.01) evolved strain (Evolved). This substantial upregulation may be attributed to the fact that the *Dof* transcription factor, sourced from soybean, may not be natively expressed in *C. pacifica*.

Initially, we evaluated the growth performance of the *Evolved Dof* against the Evolved wildtype strain to ensure that overexpression of *Dof* did not negatively impact their growth. Observations indicated no significant difference in culture density at 750 nm absorption and chlorophyll fluorescence between the transgenic and wild-type strains (Figure 2c,d; p = 0.1; n = 3; Mann−Whitney U test). Additionally, the trait for high-light intensity tolerance remained unchanged by genetic engineering (Supporting Figure 5). Next, we assessed lipid accumulation in both the evolved and evolved *Dof* strains using

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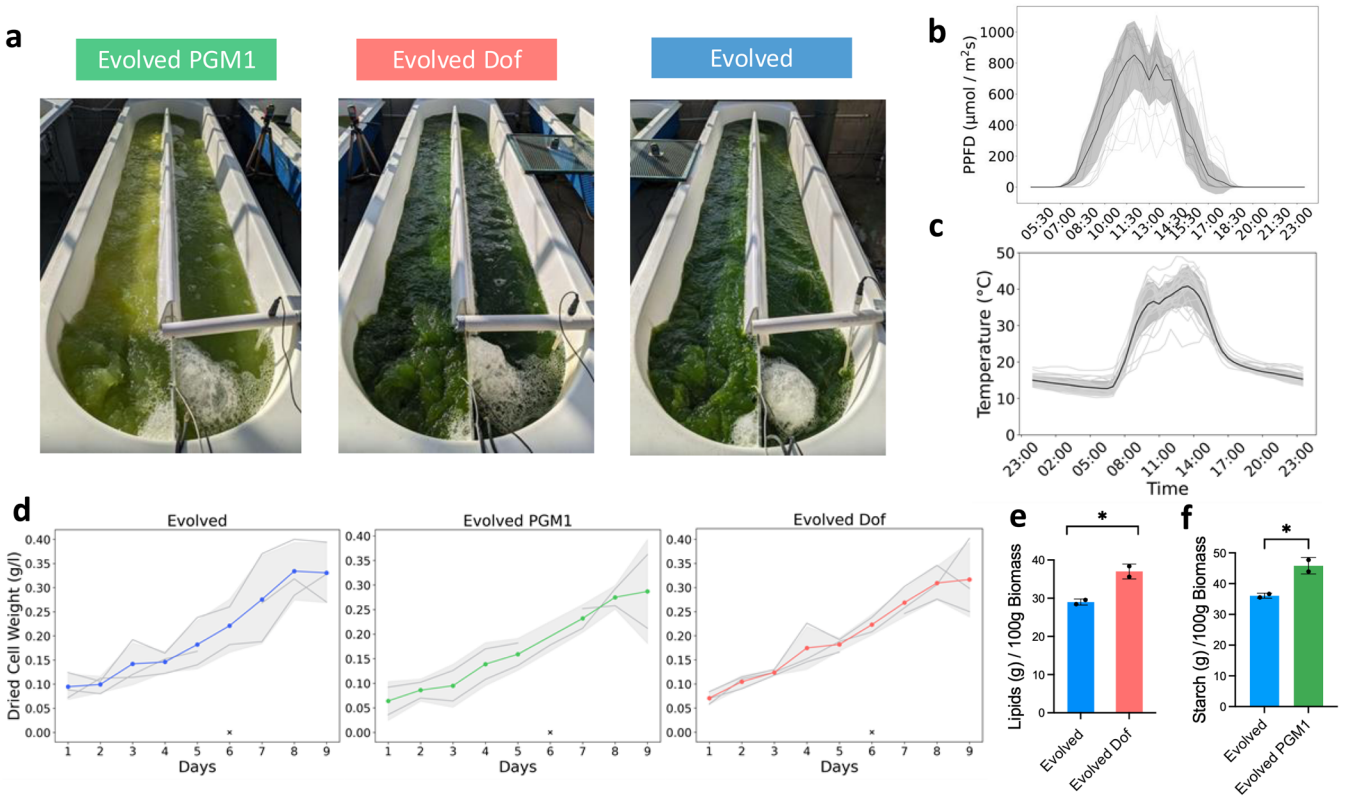

Figure 4. Pilot-scale production of high lipid and high starch strains (a) Visuals of raceway ponds showcasing cultivation of evolved and engineered *C. pacifica* strains. (b, c) Light sensor and temperature sensor data recorded during the pond runs, respectively. (d) Dry cell weight (g/L) measurements throughout three consecutive pond runs. (e, f) Lipid (g) and Starch (g) measurements relative to dried biomass (g), respectively. In panel (b), PPFD represents photosynthetic photon flux density, and in panels (b, c), a break in the line represents loss of sensor data. In panel (d), "*x*" corresponds to the day of partially missing data. In panel (e), * denotes $p < 0.05$. The *p*-values were calculated using an unpaired *t* test.

Nile Red lipid-binding dye and flow cytometry in the phycoerythrin (PE) channel under both normal and nitrogen-deprived minimal media conditions (Figure 3a). To compare the two samples, we measured the population percentage above a threshold of 10^5 RFU (Relative Fluorescence Units). About 1.2% (±0.18) of the evolved population in normal media exceeded this threshold, while the Evolved *Dof* population was approximately 29.8% (±6.62). Under nitrogen-deprived conditions, the evolved population increased to 24.1% (±4.54), and the Evolved *Dof* population rose to 47.1% (±5.05). These results indicate that the Evolved *Dof C. pacifica* strain induced more lipid accumulation under normal media conditions compared to the evolved strain, with an even more pronounced effect under nitrogen stress. Confocal microscopy further supported our flow cytometry data, revealing higher lipid-producing cells in the Evolved *Dof* strain compared to the evolved strain under both media conditions (Figure 3b). Based on these microscopy observations, we suspect that these lipid droplets are triglycerides (TAGs), as the *Dof* transcription factor is known to enhance TAG content in other microalgal species.[53,56] Collectively, these findings demonstrate that current metabolic engineering strategies using transcription factors can effectively enhance cellular lipid productivity in the evolved *C. pacifica* strain.

High Starch. Phosphoglucomutase (PGM) is an enzyme that catalyzes the interconversion of glucose 1-phosphate (G1P) and glucose 6-phosphate (G6P).[62] In higher plants such as *Arabidopsis*, *Nicotiana sylvestris*, and *Pisum sativum*, a deficiency in PGM activity results in a starchless phenotype.[63−66] In alga *C. reinhardtii*, mutants with high starch accumulation have been found to exhibit an increase in phosphoglucomutase 1 (PGM1) expression.[67] These studies indicate that PGM is central in regulating starch accumulation in higher plants and microalgae. Given the pivotal role of PGM in starch accumulation, we overexpressed *C. reinhardtii* PGM1 in the evolved *C. pacifica* strain to increase starch accumulation (Figure 2a,b). The RT-qPCR analysis revealed a 15.5-fold relative abundance in Evolved PGM1 (Cq = 31.1 ± 0.79) compared to Evolved (Cq = 36.29 ± 3.34). The relative abundance of PGM1 transcripts might be attributed to the presence of an endogenous PGM1 gene (anno1.g9484.t1) in *C. pacifica*, which shares 84% sequence identity with the PGM1 gene in *C. reinhardtii*. Similar to Evolved and Evolved Dof, Evolved PGM1 retained the trait for high-light intensity tolerance (Supporting Figure 5). And like Evolved *Dof*, we observed no negative impact on growth in Evolved *PGM1* compared to Evolved (Figure 2c,d; *p*-value = 0.4; $n = 3$; Mann−Whitney *U* test).

Next, we used the Lugol iodine staining method to observe intracellular starch.[68] This method exploits the helical structure of starch, which traps iodine molecules, resulting in a purple-black coloration. Under Differential Interference Contrast (DIC) microscopy, we observed a higher intracellular black coloration in the evolved PGM1 cells compared to the evolved strain under sulfur starvation (Figure 3c). Additionally, in the plate reading assay, we noted a 30% higher normalized absorbance at a wavelength of 660 nm (Supporting Figure 6).

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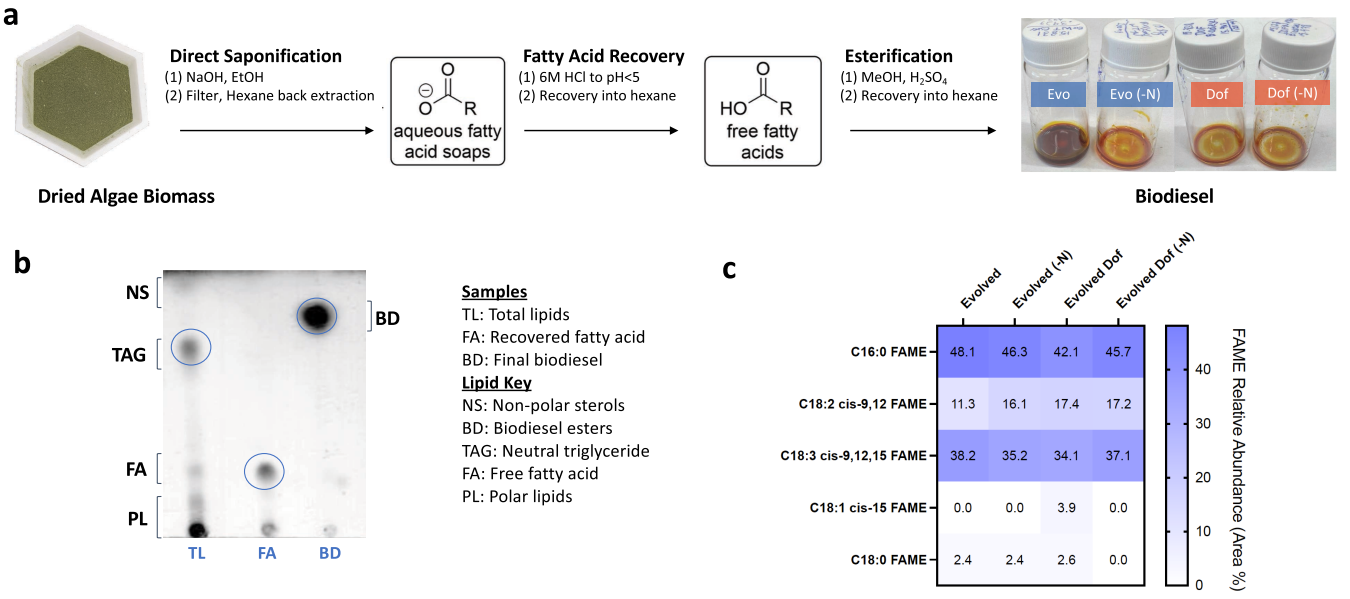

Figure 5. Conversion of algal biomass to biodiesel (a) Scheme of the conversion process from algal biomass to biodiesel. (b) Representative TLC analysis of biodiesel production process (Dof sample shown). (c) Heatmap showing GC-MS analysis of biodiesel content (n = 1). In panels (a, c), the nitrogen-deprivation condition is denoted with (−N); otherwise, the samples were in normal minimal media. In panel (c), FAME stands for fatty acid methyl esters.

Altogether, these results indicate that the PGM1 overexpressed evolved strain accumulates more starch than the evolved strain.

Pilot-Scale Cultivation of High-Lipid and High-Starch Evolved Strains. Scaling lab strains to larger-scale production in ponds often results in culture failures due to operational issues, human error, biological contamination, or environmental factors. These challenges pose significant barriers to transitioning lab strains to industrial-scale applications.[69−72]

To assess the scalability and commercial viability of our genetically engineered high-lipid and high-starch evolved *C. pacifica* strains, we initiated pilot-scale cultivation trials of the evolved, evolved *Dof*, and evolved PGM1 transgenic strains in 80 L open raceway ponds within a greenhouse environment (Figure 4a). We initially cultured the strains in 250 mL flasks containing High-Salt Media with acetate (HA) media, subsequently scaling them up to 20 L carboys under controlled laboratory conditions (see Methods section). The biomass from the carboys was then used to inoculate the 80 L ponds, which were operated photoautotrophically. We conducted three successive rounds of biomass production in closed greenhouse ponds located in San Diego, California, during November and December. Throughout the process, we maintained a high pH and closely monitored key growth parameters, including light intensity and air temperature. The ponds shown in Figure 4a are from the end of round 1 growth. Figure 4b,c display the average daily light (measured in PPFD, photosynthetic photon flux density) and average daily temperature (measured in degrees Celsius), respectively. Remarkably, given the greenhouse effect, there were days when the temperature inside the greenhouse reached up to 50 °C, demonstrating the resilience of these strains in a more natural and unconstrained setting.

We also measured the dried cell weight (g/L) of the three strains to assess biomass productivity, which reached up to 0.4 g/L in 8−9 days (Figure 4d). The maximum daily biomass productivity for *Evolved*, *Evolved Dof*, and *Evolved PGM1* were 3.90 ± 1.43, 3.38 ± 1.74, and 2.83 ± 1.16 g/m^2/day, respectively. Interestingly, we observed stagnation or no growth in the PGM1 strain in the first round. This might be due to human error or potential contamination from the inoculum prepared in minimal media supplemented with acetate as the carbon source (see Methods section). Despite this initial challenge, the *PGM1* strain exhibited a remarkable recovery in subsequent rounds, achieving growth and productivity comparable to the wildtype and the *Dof* strain, underscoring its resilience and adaptability.

We also performed gravimetric analysis on lipids and starch extracted from biomass grown in ponds to determine if the high-lipid and high-starch phenotype is consistent at scale. In the evolved Dof strain, lipid content was approximately 28% higher (p = 0.032, unpaired t test) compared to the evolved strain, with lipid content values of 28.99 ± 0.78/100 g dried biomass and 37.00 ± 1.93/100 g dried biomass, respectively (Figure 4e). Moreover, we observed a 27% increase in starch content (p = 0.039, unpaired t test; evolved: 36.08 ± 0.8/100g dried biomass, evolved PGM1: 45.83 ± 2.68/100 g dried biomass) in the evolved PGM1 strain compared to the evolved strain (Figure 4f).

Altogether, these findings collectively affirm the potential of the engineered *C. pacifica* strains to thrive in large-scale operations. Their demonstrated resilience to extreme environmental conditions, coupled with their recovery and sustained productivity, positions these strains as promising candidates for commercial-scale cultivation, offering a tangible pathway toward the production of sustainable bioproducts.

Bioproducts. *Biodiesel.* Oleaginous microalgae have been targeted as a promising feedstock for the production of renewable biodiesel. However, challenges in scalability and product purity associated with lipid extraction and chemical conversions into fuel have limited this application thus far.[73] Traditional approaches for renewable biodiesel production begin with lipid extraction and recovery from biomass to generate a total lipid extract that can be chemically converted to fatty acid methyl esters. Still, lipid extraction methods pose

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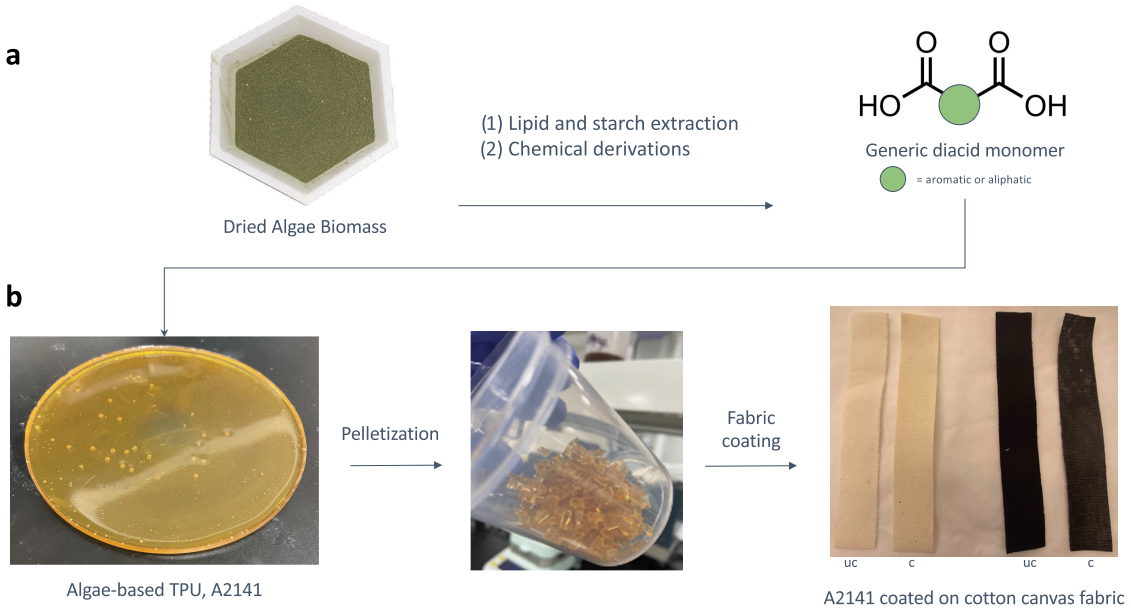

Figure 6. Conversion of algal biomass to TPU-coated fabric (a) Conversion of algal biomass to polyurethane (PU) precursors. (b) Thermoplastic polyurethane, A2141, coated on cotton canvas. (uc: uncoated, c: coated).

challenges in efficiency and efficacy.[74] Additionally, while microalgae produce high amounts of neutral lipid TAGs that can be converted into biodiesel, other classes of lipids, including nonpolar sterols and polar glycolipids, are present in total lipid extracts and can act as pervasive contaminants during biodiesel synthesis.[74]

To avoid the difficulties associated with traditional lipid extraction-chemical conversion methods of biodiesel production, we have successfully used a two-step direct-saponification esterification method to afford high-purity biodiesel directly from algal biomass.[75] In this process, dried biomass is treated with basic sodium hydroxide to selectively convert neutral triglycerides and free fatty acids within the algal cell into water-soluble charged sodium soaps (Figure 5a). This aqueous solution is extracted with hexane solvent to remove remaining uncharged lipid contaminants (nonpolar and polar lipids), enabling selective isolation of relevant biodiesel precursors. Treatment of the aqueous layer with hydrochloric acid to pH < 5 serves to reprotonate fatty acid soaps and form free fatty acids, which can then be easily recovered in an organic solvent. An aliquot of free fatty acids was saved for further characterization, and the remaining sample was converted into fatty acid methyl ester (FAME) biodiesel with methanol and sulfuric acid (Figure 5a). All four samples, including evolved and evolved *Dof* under minimal and nitrogen-deprived media, of *C. pacifica* produced yellow liquid biodiesel ranging from 3−12% of the initial cellular dry weight.

The purity of intermediate free fatty acids and final biodiesel was monitored using thin layer chromatography (TLC) with a specialized eluent and staining system that can visualize different lipid classes.[76] An example TLC of the *Dof* intermediate and product is shown in Figure 5b. When compared to a standard of total lipids extracted from *C. pacifica*, the intermediate free fatty acids and final biodiesel products displayed high purity, exemplified by the presence of only one major product spot in the TLC. The biodiesel sample showed a significant product with a retention factor (Rf) value of 0.83 compared to the fatty acid spot with an Rf of 0.25 and the TAG spot seen in the total lipids with an Rf of 0.70. This aligned with the expectations that the biodiesel product would be less polar than the initial TAGs and fatty acids and thus have a higher retention factor through TLC.

To confirm the successful synthesis of biodiesel, gas chromatography-mass spectrometry (GC-MS) was used to analyze and identify the components of each sample. Final biodiesel samples were diluted into hexane for injection, and the GC retention times and MS mass-to-charge peaks were used to identify methyl ester biodiesel products. Products from all four algal cultures displayed high purity (>80%) of mixed methyl ester biodiesel. The primary component of these mixed biodiesel samples was hexadecenoic acid methyl ester (C16:0 FAME), ranging from 42−48% relative abundance compared to the FAME mixture (Figure 5c). Other saturated (methyl stearate, C18:0 FAME) and unsaturated (C18:2 cis-9,12 FAME, C18:3 cis-9,12,15 FAME) biodiesel components were also observed in all samples. Overall, the ease of this method for producing microalgal biodiesel combined with the high purity seen in end products demonstrates the promising capability of *C. pacifica* as a producer strain of high-value industrial products.

Thermoplastic Polyurethane. To showcase the potential of the *C. pacifica* strain in practical applications, we successfully formulated thermoplastic polyurethanes (TPUs) using downstream processing of its algal biomass. We chose to design TPUs for two main reasons. First, the inherent production capabilities of the strain to produce starch and fatty acids, both of which can be converted into useful diol, diacid, and diisocyanate monomers—essential building blocks for polyurethanes.[77] Second, polyurethanes account for roughly 10% of global plastic production and are ubiquitous in our everyday lives, finding applications in coatings, adhesives, foams, and more.[78]

The synthesis of an algae-based TPU, designated as A2141, was achieved using Evolved *C. pacifica* as the main renewable source (Figure 6a,b). First, the synthesis of an algal polyester polyol was prepared via a polycondensation reaction, as

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previously reported.[79,80] The resultant polyol had a molecular weight of 1261 g/mol by hydroxyl titration and a polydispersity index (PDI) of 1.68 by gel permeation chromatography (GPC). The algal polyol was reacted with an algae-derived aliphatic diisocyanate and cured in an oven at 80° for 3 days. Once cured, the TPU had a molecular weight of 91 kDa and a PDI of 2.58 by GPC. A2141 exhibited a shore A hardness of 75 ± 3, tensile strength of 3.22 MPa, and an elongation of 282% at break.

To demonstrate possible applications, A2141 was coated onto cotton canvas fabric with a set thickness of 0.05 mm using a solution of 18% A2141 in dimethylformamide (DMF). The coated cotton fabric displayed hydrophobic characteristics with a water contact angle of 97.99 ± 0.5°, which is within the range of hydrophobic materials such as poly(ethylene terephthalate) (PET) commonly found in water bottles and polypropylene (PP) used in the packaging industry, which has contact angles of 91.3 and 105°, respectively.[81,82] The material stiffness, or flexural rigidity (G), was also analyzed by comparing the flexibility of the uncoated cotton and the algae-TPU-coated cotton. We observed a 12-fold increase in flexural rigidity after the coating treatment. The flexural rigidity was determined to be 329 mg·cm for the uncoated fabric and 4058 mg·cm for the coated fabric, thus reinforcing the fabric and contributing to the hydrophobicity of the material.

These results indicate that a 75% algae-based material could have significant commercial relevance in applications such as the fashion industry, particularly for waterproofing fabrics used in raincoats and duffle bags. Additionally, they highlight the importance of engineering robust algae strains for plastics production.

DISCUSSION

The potential of microalgae as biofactories has already been demonstrated by their ability to produce a diverse range of bioproducts, including biofuels, nutraceuticals, and biobased chemicals.[11−15,39] However, large-scale cultivation of microalgae continues to present significant challenges, primarily due to uncontrolled growth conditions, the risk of contamination, and pond crashes.[26] Our lab recently discovered a novel extremophile, *C. pacifica*, demonstrating remarkable growth resilience under high pH, high temperature, and high salinity conditions.[41] Through mutagenesis and mating, we have further evolved this strain to tolerate high light intensity, making it a robust candidate for open pond cultivation. The mating step during laboratory evolution might have altered the ploidy of the cells from haploid to diploid. However, we suspect that the subsequent generation of the Evolved *C. pacifica* strain reverted to haploid, as indicated by the presence of vegetative or gamete cells observed in the microscopy images (Figure 3). Nonetheless, future studies could focus on identifying the quantitative trait loci (QTLs) in Evolved *C. pacifica* that are associated with high light tolerance to gain a deeper understanding of the genes involved in this adaptation.

C. pacifica, belonging to the same genus as the well-studied *C. reinhardtii*, allows for the opportunity to leverage existing research on *C. reinhardtii* to accelerate the development of *C. pacifica* as an industrial strain. Methods to enhance bioproduct productivity in *C. reinhardtii* can be directly applied to *C. pacifica*. In this study, we improved lipid and starch content in evolved *C. pacifica* by overexpressing the soybean *Dof* transcription factor and the PGM1 gene from *C. reinhardtii* and demonstrated significant product enhancements (Figures 3 and 4). Similar genetic engineering strategies, such as overexpressing chloroplast-type glyceraldehyde-3-phosphate dehydrogenase (cGAPDH) to enhance overall biomass productivity or utilizing hybrid breeding approaches with selection-enriched genomic loci (SEGL) to boost photosynthetic productivity under diverse conditions could be used to further streamline the development of *C. pacifica* into a robust, high-yield strain.[83,84]

Here, we successfully demonstrated that the evolved *C. pacifica* can be grown in pilot-scale raceway ponds, leveraging its pH tolerance to avoid contamination and crashes (Figure 4). While the evolved wildtype and evolved *Dof* strains grew well without any crashes in three consecutive rounds, the evolved PGM1 strain experienced a crash in the first round but recovered in the subsequent rounds. We suspect the initial crash was due to contamination of the inoculum, prepared under heterotrophic conditions in a medium with acetate as the carbon source at a nearly neutral pH. To mitigate any future contamination risks, preparing the inoculum at a higher pH and using CO_2 captured through direct air capture technologies could be valuable.[85] This approach may reduce contamination risk and potentially make the process a net-positive source for the bioeconomy. However, further life cycle assessment is required to validate this cultivation method.[86] Moreover, given the growing interest in sustainable aviation fuel (SAF) within the airline industry, it would be highly beneficial to test the conversion of lipids extracted from the engineered strain of *C. pacifica* into SAF.[87,88] Additionally, performing a Techno-Economic Analysis (TEA) would provide a comprehensive understanding of *C. pacifica's* potential as a viable biofactory for producing SAF.

Even though, our experiments were conducted in an 80 L raceway pond, representing a small-scale model of larger production systems. Scaling up any strain presents new challenges, particularly in large-scale open raceway systems, where longer raceways and varying mixing levels impact light availability, nutrient uptake, and gas exchange.[89] Economic feasibility is also a key factor for scalability, which has yet to be demonstrated for this new species. Biomass productivity plays a crucial role in determining feasibility, but a more thorough evaluation through a TEA is needed to identify areas for optimization. Previous TEAs on other species have highlighted the importance of biomass productivity and lipid content.[90] Another crucial factor along the same lines is year-round productivity, which was outside the scope of this study but remains an important consideration. *C. pacifica* likely exhibits seasonal variations in productivity, suggesting that its cultivation may be more suited to certain seasons. Rotating it with other strains could enhance overall farm productivity while also disrupting the buildup of strain-specific pathogens, potentially protecting other algae species in the system.[69,91]

One further challenge is the use of recombinant strains. While our experiments were conducted in a controlled greenhouse environment, large-scale production would require fully open systems, raising concerns about potential strain escape. However, an environmental study using genetically modified *C. reinhardtii* showed no spread of the recombinant strain into surrounding environments over the study period, suggesting that similar risks for *C. pacifica* may be manageable, though further investigation is warranted.[92]

Another key consideration is that most studies have primarily focused on extracting a single valuable feedstock, such as lipids, carbohydrates, or proteins, from algal biomass.

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To enhance the economic viability of algae, it would be beneficial to optimize downstream extraction processes to efficiently extract all proteins, carbohydrates, and lipids simultaneously.[93,94] These can then be converted into valuable products such as therapeutic proteins, animal feed, biomaterials, and biofuels. This comprehensive approach would reduce the burden and high cost associated with a single commodity and help integrate algae into the mainstream of biotechnology. *C. pacifica* is an excellent candidate for such tests, given its compatibility with genetic engineering and large-scale cultivation.

CONCLUSIONS

This study demonstrates the potential of developing extremophile microalgae as a commercially viable production platform. Here, we have demonstrated the successful transformation of the novel extremophile species, *C. pacifica*, into a robust, high-yield strain. Additionally, pilot-scale cultivation has shown the feasibility of producing biobased fuel and material, highlighting the potential for large-scale applications. By enhancing its resilience, employing genetic engineering, and achieving large-scale growth, we have demonstrated *C. pacifica's* potential to produce valuable bioproducts such as biofuels and polyurethane. The versatility of *C. pacifica* as a biofactory highlights the promise of extremophile algae in a future sustainable bioeconomy, offering potential solutions to address climate change and global resource demands. Future research should focus on optimizing downstream extraction processes and conducting comprehensive techno-economic analyses to fully realize the potential of *C. pacifica* for industrial applications.

METHODS

Media and Algae Strain. For lab flask cultures and 20 L carboys, High-Salt Media with acetate (HA) was used, with or without high pH.[41] If the media had a high pH, NH_4Cl was replaced with urea, and the pH was raised to 10.5 to avoid the release of NH_3. For nitrogen-deprived media, the nitrogen source (NH_4Cl or urea) was removed. Ammonium nitrate was used as a nitrogen source for pond pilot runs. For pond main runs, urea was used as the nitrogen source, and the pH was increased and maintained daily at 10.5 using NaOH. The medium contained 40−100 mM NaCl and no additional carbon source, thus operating under fully phototrophically conditions.

C. pacifica (CC-5699) and *Evolved C. pacifica* (CC-6190 402 wt × 403 wt) were used throughout all experiments, which can be ordered from Chlamydomonas Resource Center at the University of Minnesota in St. Paul, Minnesota.[41] In the lab, the cells were grown under continuous 24 h light conditions at approximately 25 °C, with a photon flux of 125 $\mu E/m^2/s$, on shaken tables rotating at 125 rpm.

Mutagenesis and Breeding. To induce mutagenesis, we first established a UV radiation killing curve to determine the optimal exposure time that resulted in significant cell death while leaving some viable cells. Cells were grown in HA medium to a density of $(1-2) \times 10^7$ cells/mL, with 5 mL of culture per 150 mm Petri dish, maintaining a liquid culture height of ∼3 mm to minimize UV absorbance by the medium. UV exposure was performed using a BioRad GS Gene Linker UV Chamber with 5-s intervals of continuous light. After each interval, samples were plated on HA agar and incubated for 7 days to assess survival. The optimal exposure time, where significant cell death occurred with remaining viable cells, was found to be 35−40 s.

To generate a mutant library, stationary-phase *C. pacifica* 402 cells were exposed to UV light for 35−40 s, as determined by the killing curve. Postexposure, cells recovered for 24 h in fresh media under standard culturing conditions. During the screening phase, cells were plated on high-light screening plates composed of High Salt media (HSM) media agar and exposed to varying light intensities. The light gradient was achieved by placing the plates 3 cm from a 1000W Mastiff Growl LED Grow Light (HERB PRO G2) and applying shading filters with different opacities (0, 25, 50, 75%) (Supporting Figure 1). After 24 h of light exposure, plates were incubated at 80 $\mu E/m^2/s$ until colonies formed. Cells at the edge of light tolerance were scraped, allowed to recover for 24 h, and replated on HA agar.

For breeding, we followed the protocols from Findinier and Molino et al., using wildtype *C. pacifica* 403 to mate with the mutant *C. pacifica* 402 cell library.[41,95] Initially, both strains were grown separately on HA agar for 7 days, then harvested and resuspended in nitrogen-deprived HA medium. After overnight exposure to 80 $\mu E/m^2/s$ light, the strains were mixed and incubated in low light (∼8 $\mu E/m^2/s$) for 24 h. Mating was confirmed microscopically by observing characteristic mating cues.[41] The mixture was centrifuged at 2000*g* for 5 min, resuspended in fresh media, and returned to standard growth conditions. The mated cells were screened using high-light screening plates, and colonies at the tolerance edge were selected. The pool of high-light-tolerant colonies was subjected to mutagenesis and subsequently spread across a pH-gradient plate. A colony from the high-pH region was selected as the evolved strain for further analysis. This strain was compared to the parental line using a high-light tolerance assay by spreading both lines on the same HSM agar plate and exposing them to the light gradient. Tolerance was assessed based on survival across the light gradient, with higher tolerance attributed to strains that survived in regions of greater light exposure.

High Light Tolerance Assay. The strains were exposed to high light conditions, ranging from a minimum of 500 $\mu E/m^2/s$ to a maximum of 3000 $\mu E/m^2/s$, over a continuous 24 h period, followed by incubation at 80 $\mu E/m^2/s$ for 5 days. The gradient of light exposure is visually represented by the decreasing density of algal growth from the bottom (500 $\mu E/m^2/s$) to the top (3000 $\mu E/m^2/s$) of each plate. The gradient was generated by adding a strong light source (HERB PRO G2, 1000W Mastiff Growl LED Grow Light) with blue and red LEDs 9 cm from the plates. Positioning the plates on the corner of the panel allows for a linear gradient light intensity measured with a photometer (Supporting Figure 3).

High pH Tolerance Assay. The cells were grown up to late exponential to stationary phase and were transferred to high pH media for 24 h. After 24 h, cells were plated on HA agar and grown for 6−7 days in standard conditions.

Vector Design and Algae Transformation. The vector was constructed using genetic elements derived from the assembled genome of *C. pacifica*. The β-Tubulin A 2 gene promoter was utilized to drive the expression of hygromycin (used as a selection marker) and the *Dof* or *PGM1* gene. The complete map can be found at Zenodo (10.5281/zenodo.12636981). The Dof (pJPCHx1_Dof_1.1) and PGM1 (pJPCHx1_PGM1) vectors have been submitted to the

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Chlamydomonas Resource Center at the University of Minnesota in St. Paul, Minnesota.

For the transformation process in algae, plasmid DNA was digested using *Kpn*I and XbaI enzymes (New England Biolabs, Ipswich, MA). This was followed by purification with the Wizard SV Gel and PCR Clean-up System (Promega Corporation, Madison, WI) without separating the fragments. DNA concentration was measured using the Qubit dsDNA High Sensitivity Kit (Thermo Fisher Scientific, Waltham, MA). Electroporation was used for transformation, following the method described by Molino et al..[96] Transformed cells were selected on HA agar plates containing 30 μg/mL hygromycin.

The transformants were verified through Colony-PCR using primers specific for a 277 base pair segment of the hygromycin gene (forward primer: TGATTCCTACGCGAGCCTGC; reverse primer: AACAGCTTGATCACCGGGCC), followed by sequencing.

Reverse Transcription-Quantitative Polymerase Chain (RT-qPCR) Reaction. To assess the abundance of Dof or PGM1 transcripts, total RNA was first extracted from samples using the Direct-zol RNA Miniprep Plus Kit (Zymo Research). cDNA synthesis was then performed with the AzuraFlex cDNA Synthesis Kit (Azura Genomics), utilizing a mix of random hexamers and oligo(dT) primers to achieve comprehensive coverage of all RNA transcripts.

Quantitative PCR (qPCR) was conducted using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) on a Bio-Rad thermocycler. The qPCR protocol consisted of an initial denaturation and polymerase activation step at 98 °C for 3 min, followed by 40 cycles of amplification with denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. A melt curve analysis was performed from 65 to 98 °C, with temperature increments of 0.5 °C every 5 s. ATP2 was used as the housekeeping gene for normalization. The relative abundance of target genes was calculated using the $2^{-\Delta\Delta CT}$ method.[97] The primers used for amplification were: ATP2 (housekeeping gene)—Forward: 5′-ACGGTGGTTTCTCTGTGTTC-3′, Reverse: 5′-CACACCCGACTCAATCATCTC-3′; Dof—Forward: 5′-CCTCTACTGCATGTACGGTAAC-3′, Reverse: 5′-TGGTCGCGTTGCTCATAAT-3′; and PGM1—Forward: 5′-GGAGAACGACTTCGGCATTA-3′, Reverse: 5′-GACAGATCGACGTCAGGAATATC-3′.

Plate Reader Assay. A volume of 160 μL from each sample was placed in a flat-bottom 96-well plate (Corning Costar, Tewksbury, MA). Fluorescence was measured using a Tecan Infinite M200 Pro plate reader (Tecan Group Ltd., Männedorf, Zurich, Switzerland). Chlorophyll fluorescence was recorded with an excitation wavelength of 440 nm and an emission wavelength of 680 nm, while absorbance was measured at a wavelength of 750 nm.

Nile Red Staining and Flow Cytometry. A 50 μL aliquot of culture was diluted with 110 μL of buffer to reach a final volume of 160 μL. Then, 1.6 μL of 1 mM Nile red in DMSO was added to the solution. The cells were incubated at 40 °C for 10 min using the Tecan Infinite M200 Pro plate reader (Tecan Group Ltd., Männedorf, Zurich, Switzerland). The stained and incubated samples were analyzed using a Beckman Coulter CytoFLEX Cytometer, utilizing the PE channel (585/42 bandpass filter) to detect Nile Red fluorescence (excitation/emission 551:636 nm) bound to intracellular lipids with a gain setting of 1. 10,000 events were recorded at a 30 μL/min flow rate for each sample.

Microscopy. For Differential Interference Contrast (DIC) microscopy, cells were stained with 1% Lugol's solution and imaged using a Nikon Eclipse Ni microscope. Images were taken at 60× magnification plus the ocular lens and the same gain and exposure settings were maintained for all samples to ensure consistency.

For confocal microscopy, sample slides were prepared using Frame-Seal Slide Chambers containing agar supplemented with media following the protocol described in https://www.protocols.io/view/agarose-pads-for-microscopy-kxygxer6wv8j/v1. Cells were placed on the agar and sealed with a glass coverslip. Confocal microscopy was performed at the UCSD School of Medicine Microscopy Core using a Leica STED microscope. An excitation wavelength of 405 nm with an emission range of 630−680 nm was used for chlorophyll detection. An excitation wavelength of 470 nm with an emission range of 475−575 nm was utilized for Nile red. The emission and excitation settings were carefully adjusted to minimize channel cross-talk.

Raceway Ponds. Three 80 L raceway ponds were utilized for algae cultivation inside a greenhouse with each having a surface area of 1.75 m^2. The ponds were filled with HSM prepared using normal tap water. For pilot runs, we used ammonium nitrate as the nitrogen source. However, urea served as the nitrogen source for later main runs, and the pH was elevated to 10.5 using NaOH. The pH was monitored and maintained at 10.5 daily. The media contained 40 mM NaCl and excluded any additional carbon source, enabling fully phototropic growth. The ponds were exposed to natural sunlight, and algal growth was monitored daily by measuring chlorophyll (440:680), absorbance at 750 nm, and dry cell weight (DCW, g/L). The greenhouse was equipped with sensors to monitor culture pH, culture temperature, light intensity, and air temperature throughout the experiment. For DCW measurement, a 50 mL sample was collected and initially centrifuged at 3000*g* for 5 min. The pellet was then resuspended in a 50 mM EDTA solution at pH 8, and this process was repeated twice to effectively remove salts. Afterward, the pellet was resuspended in water to eliminate any residual EDTA, followed by a final centrifugation step before drying the sample in an oven to determine the dried biomass weight.

Lipid Extraction. Lipid extraction was carried out on the evolved and evolved Dof samples from the pilot run, each having two technical replicates. A 50 mL culture was centrifuged and dried. The dried biomass was then subjected to the Bligh−Dyer lipid extraction method.[98] The extracted lipids were weighed, and the data was presented as a percentage of the dried biomass.

Starch Extraction. Following lipid extraction, the residual biomass was subjected to several cycles of extraction using 80% ethanol and 50 mM NaOH to remove any remaining protein and lipid content. This process was repeated until a white sediment was observed. The sediment was then washed twice with water to remove any residual ethanolic solution and subsequently dried. The dried biomass extract was subjected to lugol and anthrone colorimetric determination against calibration curves of known starch quantities for each test respectively, in addition to FTIR for purity analysis (Supporting Figure 7). The extraction method provided starch granules with >90% purity.

Conversion of Algal Biomass to Biodiesel. *Biodiesel Synthesis.* Algal biodiesel was prepared via direct biomass

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saponification followed by acid-catalyzed Fischer esterification.[99] Wet algal biomass slurry samples were flash frozen in a dry ice/isopropanol bath prior to lyophilization to sublimate excess aqueous media (LabConco FreeZone 2.5, 0.6 mbar reduced pressure, −52 °C). The masses of dried samples were recorded prior to mechanical grinding with a mortar and pestle. Ground algal powder was stirred at 500 rpm in a 95% ethanol solution (1 mL solvent: 1 g dry algae) for 15 min before saponification. Solid sodium hydroxide (30% w/w NaOH/dry algae) was added to the solution, and saponification was carried out under reflux at 80 °C for 2.5 h. Following reflux, the hot saponification solution was vacuum filtered to remove remaining solid biomass and the filtrate was allowed to cool overnight to form a solution of solid sodium soaps. Deionized water was added to the filtrate to dissolve soaps, and the mixture was washed with hexane to remove unsaponifiable material (carotenoids, sterols, etc.). Sodium soaps were converted to free fatty acids via protonation with 6 M HCl (pH < 5, stir 1.5 h, rt), and the resulting product was extracted into hexane and dried under rotary evaporation. Free fatty acids (FFAs) were esterified under acidic conditions to produce algal biodiesel (1:4 w/v FFA/MeOH, 0.4 equiv H_2SO_4, 80 °C, 2 h). The final reaction mixture was extracted into hexane and washed with methanol to remove trace remaining FFAs. The solvent was removed under the airstream to yield orange liquid biodiesel.

Biodiesel Analysis. The final biodiesel products were characterized using TLC and GC-MS. Samples of final biodiesel, intermediate free fatty acids, and a reference of *C. pacifica* total lipids prepared from a continuous hexane extraction were analyzed by TLC using glass-backed silica gel 60 plates with a 70/30/1 hexane/diethyl ether/acetic acid eluent. Plates were visualized with a cupric sulfate stain (10% (w/v) $CuSO_4$, 4% (v/v) H_2SO_4, 4% (v/v) H_3PO_4 in MeOH) and charring at 160 °C.[76]

GC-MS analysis of biodiesel was performed on an Agilent 7890A GC system connected to an Agilent 5977C GC/MSD. Samples were separated on an Agilent HP-5MS UI 30 m × 0.25 mm × 0.25 μm GCMS column with hydrogen as carrier gas and a gradient of 3 °C/min from 70 to 250 °C over 60 min. Integration values from associated peaks were used to determine the relative abundance of different FAMEs comprising each biodiesel sample.

Conversion of Algal Biomass to TPUs. The 75% algae-based TPU material was prepared as previously described utilizing a polyol.[79,80,100]

The synthesis involved creating a polyester polyol composed entirely of algae-derived aromatic and aliphatic diacids from *C. pacifica* with a renewable linear diol, which was then reacted with a linear algal diisocyanate to afford the algae TPU, A2141. A2141 had mechanical properties tested in accordance with the American Society for Testing and Materials (ASTM) under tests D2240 and D624-00 for hardness and tensile strength, respectively. Gel permeation chromatography (GPC) was carried out on a Malvern GPC system with a Tosoh TSKgel SuperHZM-N and guard columns with molecular weights and distribution relative to a polystyrene standard. Tetrahydrofuran (THF) served as the polymer solvent and eluent. A2141 was dissolved in DMF at a concentration of 18% and applied to cotton canvas fabric using a coating spreader set to a thickness of 0.05 mm. Water contact angle was tested on a Goniometer, ramé-hartModel 200, and stiffness was in accordance with ASTM D1388-18.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestengg.4c00443.

Light filter used; photographs of selection plates during in vitro evolution; a curve illustrating the light gradient on high-light selection plates; microscopy images of the evolved *C. pacifica* strain cocultured with two *C. pacifica* mating types; data on light intensity tolerance of transgenic strains, absorbance measurements of Lugol-stained evolved PGM1 strain compared to evolved strain, and an analysis of the starch extraction method (PDF)

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Notes

The authors declare the following competing financial interest(s): Stephen Mayfield and Michael D. Burkart are cofounders of and holds equity in Algenesis Inc., a company that could potentially benefit from this research. Marissa Tessman is an employee and shareholder in Algenesis Inc. The other authors declare that their research was conducted without any commercial or financial relationships that could be perceived as potential conflicts of interest.

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