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Development and Large-Scale Production of High-Oleic Acid Oil by Fermentation of Microalgae

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Abstract: Our classical strain improvement began with an isolate showing 28% palmitic and 60% oleic acids. UV and chemical mutagenesis enhanced our strain's productivity, carbon yield, and oleic acid content. The iterative methodology we used involved the creation of mutant libraries followed by clonal isolation, assessments of feedstock utilization and growth, oil titer, and the validation of oil composition. Screening these libraries facilitated the identification of isolates with the ability to produce elevated levels of oleic acid, aligning with the targets for high-oleic acid substitutes. Utilizing a classical strain improvement approach, we successfully isolated a high-oleic acid strain wherein the level of oleic acid was increased from 60 to >86% of total FA. The performance of the classically improved high oleic acid-producing strain was assessed at fermentation scales ranging from 1 L to 4000 L, demonstrating the utility of our strain and process at an industrial scale. These oils offer promise in various applications across both the food and industrial sectors, with the added potential of furthering sustainability and health-conscious initiatives.

Keywords: fermentation; high-oleic acid oil; microalgae; nutrition

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1. Introduction

Oil production through fermentation has historically centered on long-chain polyunsaturated (LC-PUFAs) omega-3 fatty acids [1]. However, this reflects a somewhat narrow focus, given the technological progress made over the last two decades and the burgeoning demand for alternative sources of fats and oils with fewer environmental and supply chain issues [2]. Microorganisms offer a diverse array of oil production capabilities, presenting viable alternatives to conventional fats and oils while catering to the multifaceted requirements of the industry. Among the myriad of microorganisms, yeasts and microalgae emerge as frontrunners due to their adaptability to industrial-scale cultivation and their potential as producers of oils with desirable nutritional and physicochemical attributes [2].

The successful development and implementation of fats and oils production via fermentation may represent a paradigm shift in the quest for sustainable lipid sources, with far-reaching implications for food security, environmental sustainability, and public health initiatives. In addition to addressing food industry demands, oils obtained through fermentation hold promise in applications ranging from home and personal care, to lubricants, oleochemicals, and materials.

Conventional fats and oils, once hailed as staples of modern diets, now face heightened scrutiny due to their association with health ailments and environmental concerns [3,4]. For example, tropical fats such as palm oil, once lauded for their versatility and utility, are now viewed through a critical lens as consumers, policymakers, and industries alike seek more ecologically responsible and nutritionally beneficial alternatives [3]. Microalgae, with their remarkable growth rates, high lipid content, and adaptability to various environmental conditions, offer a compelling solution to these pressing challenges confronting the fats and

oils industry [2]. Their innate ability to thrive in diverse ecological niches positions them as prime candidates for more sustainable oil production. Furthermore, the amenability of microalgae to genetic manipulation and optimization makes them ideal targets for tailoring fatty acid compositions to mimic those found in traditional oils such as high-oleic oil [5] and more recently structured lipids such as human milk fat analogues [6]. In an earlier investigation, we documented the development of a microalgal strain tailored to produce structured lipids intended for infant nutrition applications. This strain was engineered through a series of classical strain optimization steps to enhance the concentration of palmitic acid from 25 to 30% of total fatty acids [6]. Subsequently, a regio-specific lysophosphatidic acid acyltransferase (LPAAT) was integrated into the algal genome to facilitate the accumulation of palmitic acid in the sn-2 position of the resulting triacylglycerols (TAG).

Classical strain improvement refers to methods of random or semi-random mutagenesis to create non-naturally occurring strains with improved properties. While genetic engineering techniques are very effective in designing new algae-derived fats and oils, leveraging classical strain improvement techniques can fulfill the demand for non-genetically engineered fats and oils produced by fermentation. These methods include mutagenesis of a population to create genetic variants, selection or screening of a surviving population to enrich for specific classes of variants, and identification of improved variants by assaying for increased product titer, yield, or productivity. Classical strain improvement methods can include exposure to UV radiation, chemical mutagens, and/or selective or enrichment agents, and offer additional advantages when employed as the sole strategy to modify or enhance strain phenotype and performance. These benefits are evident from both a regulatory and business/marketing standpoint. Regulatory benefits include potential exemptions from oversight by regulations such as the U.S. Toxic Substances Control Act (TSCA) administered by the EPA and/or obligations to submit a Microbial Commercial Activity Notice (MCAN) for non-genetically engineered microbes intended for non-food applications. Similar exemptions exist in other regions, such as Brazil, which does not require filing a Strain Dossier with the National Technical Commission of Biosafety (CTNBio). This regulatory dispensation can result in significant cost and time savings during development. Moreover, from a marketing and consumer branding perspective, products derived from non-genetically engineered processes may be able to obtain organic and Non-GMO labeling certifications (e.g., Non-GMO Project Verified), helping meet consumer preferences for "clean labeling" and enhancing brand reputation.

In this study, we used classical strain improvement techniques to explore the potential of a *Prototheca moriformis* isolate, known for its production of oleic and palmitic acids as its primary fatty acids, to serve as a foundation for generating mutants that align with the compositional standards of conventional oils like high-oleic acid oils. Using UV and chemical mutagenesis and iterative growth and fatty acid composition assessment cycles, we meticulously interrogated mutant libraries to identify strains displaying heightened levels of oleic acid with increased oil productivity. Our ability to successfully scale production and processing highlights the viability and industrial relevance of these classically improved microalgal strains, signaling a new era of more sustainable lipid production.

2. Materials and Methods

Strain development. Classical strain improvement was initiated on the wild-type *P. moriformis* strain isolate UTEX 1533, which displayed levels of 28% and 56–60% of palmitic and oleic acids, respectively. All reagents and chemicals were obtained from Sigma-Aldrich (Sigma-Aldrich, St Louis, MO, USA). Classical strain improvement regimes included repeated rounds of UV (8000–20,000 uJoules) and chemical (ethyl methane sulfonate-EMS, 270 mM, 45 min @ 32 °C; 4-nitroquinoline 1-oxide-4-NQO, at concentrations, times, and temperatures ranging from 2.7–60 μM, 5–30 min, and 28–32 °C, respectively) mutagenesis. The enrichment strategies imposed post-mutagenesis included both chemical (Cerulenin, a b-keto-acyl-ACP synthase inhibitor at 7–50 μM; AZD, an mTOR kinase inhibitor at 26–75 μM and Clomiphene, or an inhibitor of sterol biosynthesis at 12–100 μM) and physical

(buoyant density centrifugation) means. The iterative methodology involved the creation of mutant libraries followed by clonal isolation, assessments of feedstock utilization and growth, and validation of oil composition. Screening these libraries led to the identification of isolates with the ability to produce elevated levels of oleic acid, aligning with the targets for high-oleic acid substitutes. Detailed conditions for the production of the high-oleic algae strain are provided elsewhere [7].

Culture media composition. The vegetative growth medium was comprised of macronutrients including NaH_2PO_4, K_2HPO_4, Citric acid monohydrate, Magnesium sulfate heptahydrate, Calcium chloride dihydrate, and dextrose at 1.64, 1.98, 1.05, 1.23, 0.02, and 40 g/L, respectively. Ammonium sulfate served as the sole nitrogen source at 1.0 g/L. Antifoam (Sigma 204, Sigma-Aldrich, St Louis, MO, USA) was added to a final concentration of 0.225 mL/L. Trace minerals were prepared as a 1000 X stock solution comprised of boric acid, zinc sulfate heptahydrate, manganese sulfate monohydrate, sodium molybdate dihydrate, nickel nitrate heptahydrate, citric acid monohydrate, copper (II) sulfate pentahydrate, and Iron (II) sulfate heptahydrate at 0.91, 1.76, 1.23, 0.05, 0.04, 20.49, 0.05, and 0.75 g/L, respectively. A 1000 X vitamin stock was comprised of thiamine HCl, D-pantothenic acid hemicalcium salt, biotin, cyanocobalamin, riboflavin, and pyridoxine HCl at 3.0, 0.16, 0.0048, 0.00034, 0.015, and 0.0078 g/L, respectively. The lipid production medium was identical to the vegetative growth medium, except that ammonium sulfate was supplied at 0.2 g/L. All solutions were filter sterilized prior to use.

Production of algae oil. The production process was scaled from the 1 L to the 4000 L reactor scale essentially as described in [8], followed by drying of the algal fermentation broth on a double drum dryer (Buflovak Model ADDD operating at 70 psig steam, 1200 rpm; Buflovak, Tonawanda, NY, USA), and subsequent mechanical/solvent extraction utilizing a 6:1 hexane to biomass ratio carried out for up to 6 h on an MSE Pro planetary ball mill (10 L capacity). Micellae resulting from solvent extraction were subsequently roto-vaped to remove hexane, followed by degumming using citric acid (0.2% wt:wt of a 50% solution) at 130 °C for 10 min with agitation. The removal of gums was carried out by centrifugation at 3000 rpm for 10 min, after which the de-gummed oil was decanted. This was followed by bleaching under a vacuum (27 in Hg) and high temperature (90–110 °C) using 2% (wt:wt) bentonite bleaching earth. Bleaching clay and oil were separated by filtration under vacuum followed by steam deodorization under vacuum (<25 in Hg at 200 °C for 90 min) resulting in refined, bleached, and deodorized (RBD) oil. Typical fermentation results obtained at the 1, 20, and 4000 L scales are provided in Table 1. The fermentation conditions utilized included an inoculation volume of 0.25–0.3 of the fermentor volume and pH and dissolved oxygen setpoints of 5.5–6.0 and 30%, respectively. The operating temperature was 28 °C throughout, with aeration and agitation managed to maintain DO. The fermentation medium was fortified to support higher cell density as follows. The medium composition remained the same. However, trace metals and vitamin solutions were increased 15 and 10-fold, respectively. Micronutrients (sodium phosphate, potassium phosphate, citric acid monohydrate, magnesium sulfate heptahydrate, and calcium chloride dihydrate) were increased to 7.13, 9.25, 2.1, 17.33, and 0.8 g/L, respectively. The glucose feed was a 55% wt:wt sterile solution.

Fatty acid (FA) analysis. The fatty acid compositions of the algae and high-oleic sunflower oil samples were measured as their fatty acid methyl esters (FAMEs) following direct transesterification with a sulfuric acid methanol solution [9]. Samples were injected on an Agilent 8890 gas chromatograph system equipped with a split/splitless inlet and a flame ionization detector (Agilent Technologies, Palo Alto, CA, USA). An Agilent DB-WAX column (30 m × 0.32 mm × 0.25 um dimensions) was used for the chromatographic separation of the FAME peaks. A FAME standard mixture purchased from Nu-Chek Prep (Nu-Chek Prep Inc., Elysian, MN, USA) was injected to establish retention times. Response factor corrections were determined using various standard mixtures from Nu-Chek Prep. Methyl nonadecanoate (19:0) was used as an internal standard for quantitation of individual FAMEs. Run to run reproducibility is insured by running an internal reference standard

biomass control sample. As an example, this standard, run over three years and assessing 18 random runs, shows a standard deviation in oil content (g/L) of 0.80 g/L (average of 49.14), oleic acid content of 0.13 area % (average of 83.60), total saturate content of 0.05 area % (average of 7.43), total monounsaturated FA content of 0.14 area % (average of 84.68), and total polyunsaturated FA content of 0.03 area % (average of 7.51). The coefficient of variation (CV%) across these five parameters was 1.62, 0.15, 0.73, 0.17, and 0.40, respectively.

Table 1. Key performance indicators (KPIs) for high oleic strains in 1 L, 20, and 4000 L vessels. 1 L and 20 L runs were carried out using a final nitrogen concentration of 320 mM while 4000 L runs utilized a 170 mM process. Differences reflect the lower OTR achievable in the larger vessel, necessitating a process that would put fewer cells in place to stay within the capabilities of the fermentor. Differences in 20 L and 1 L and 4000 L scales also reflect differences in vessel configuration. The 1 L and 4000 L fermentors required higher initial operating volumes and hence earlier draws, reducing final oil and DCW titers.

Fermentation Run	Oleic Acid (% of Total FA)	Dry Cell Weight (DCW, g/kg)	Oil Titer (g/L)	Oil Content (% of DCW)
Base Strain, 1 L	60.47	197.00	132.50	67.25
High-oleic acid strain				
Run 1, 1 L	87.23	207.30	137.20	66.18
Run 2, 1 L	87.47	207.90	140.20	67.44
Run 3, 1 L	87.29	211.90	145.20	68.52
Run 4, 20 L	85.35	245.10	161.20	65.77
Run 5, 4000 L	85.10	209.20	143.10	68.00
Average	86.49	216.28	145.38	67.18
Standard deviations	1.04	14.50	8.36	1.05
Coefficient of variation (%)	1.20	6.70	5.75	1.56

3. Results

3.1. Classical Strain Improvement Strategy

The fatty acid composition of the native strain of *P. moriformis* is remarkably simple, consisting predominantly of oleic acid (18:1 n-9) followed by palmitic (16:0), linoleic (18:2 n-6), and stearic (18:0) acids (see Figure 1). These fatty acids are abundantly present in daily diets through the consumption of vegetable oils (18:1 n-9 and 18:2 n-6) and animal and plant fats (16:0, 18:0, and 18:1 n-9). Furthermore, this strain exhibits remarkably high productivity, enabling industrial-scale oil production at competitive costs (Figure 2). Thus, this microorganism was used for generating strains with modified fatty acid compositions using classical strain optimization techniques. The study presented here focused on obtaining strains capable of producing oils rich in oleic acid using a novel iterative strain optimization workflow, allowing for highly efficient mutant library screening as displayed in Figure 3.

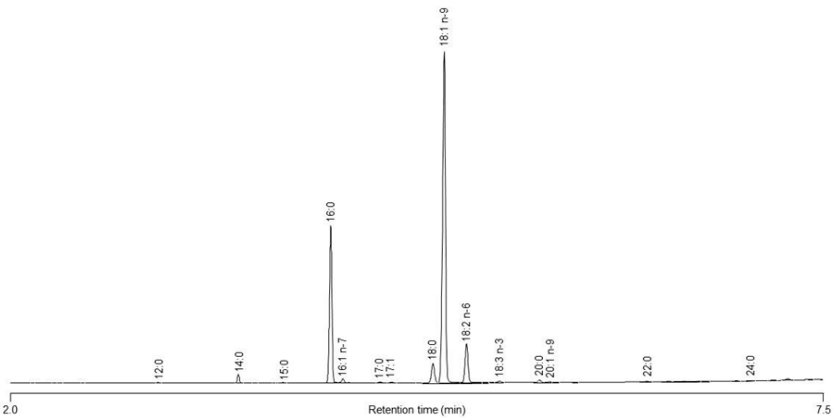

Figure 1. Gas-chromatogram of *P. moriformis* fatty acid methyl esters in the base strain.

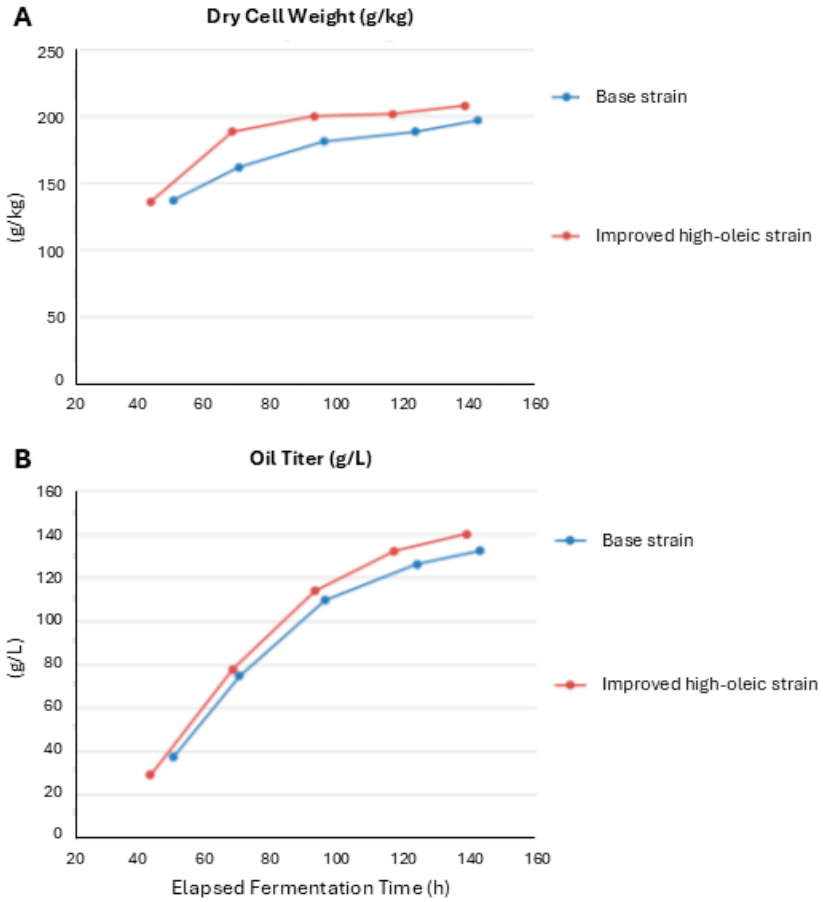

Figure 2. Comparison plots showing dry cell weight (DCW) (**A**) and volumetric oil production (**B**) for the base strain versus the classically improved high oleic strain 1 L scale.

As shown in Figure 3C, algal cells in the log phase of growth are grown in the vegetative growth medium for 24–30 h at 28 °C in a 96-well block format with shaking (Infors Multitron shaker (Axon Labortechnik GmbH, Kaiserslautern, Germany), 0.5 mL culture/well, 900 rpm) are subjected to chemical or UV mutagenesis (step 1). The cells are then sub-cultured into lipid production medium (same 96 well block format, cultivation for up to 72 h) where they are subjected to selection/enrichment strategies (step 2). The strains are then plated to a solid medium (vegetative growth medium plus 2% agar) to obtain clonal isolates (step 3), followed by the interrogation of these isolates in the lipid production medium in a 96-well format as described above (step 4). Using glucose consumption as a more rapid surrogate for oil production (the authors have found that glucose consumption tracks linearly with oil production in g/L), high glucose-consuming strains can be interrogated for their fatty acid profiles, and promising candidates can then be validated in lipid production medium in a tube or shake flask format (step 3 & 4). Isolates which are successfully validated are sub-cultured for multiple generations to stabilize their mutations, followed by the purification of clonal isolates and subsequent re-interrogation in lipid production medium (step 5). Clones deemed to be phenotypically stable are ready for further validation in fermentors (step 6), while clones that still show variability in phenotypes are passaged repeatedly until the targeted composition and strain performance characteristics are obtained in a stable line (iterative process).

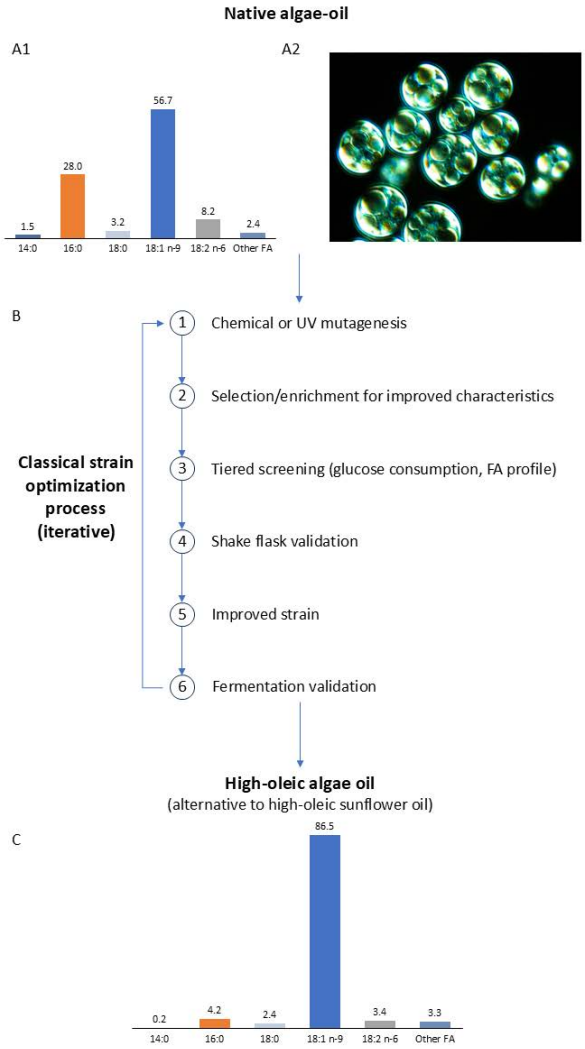

Figure 3. Fatty acid profile (**A1**) and cell morphology at the end of fermentation (**A2**) of native *P. moriformis* isolate used to produce high oleic acid algae oil (**C**) by a classical strain optimization strategy (**B**).

3.2. Development of High-Oleic Strain

The development of the high-oleic acid-producing algae strain from the parental strain involved a systematic process of classical strain improvement and mutagenesis to enhance productivity, carbon yield, and oleic acid content. The initial step involved classical strain improvement, in which cells were exposed to UV light to induce random mutations. This was followed by selection strategies to isolate high glucose-consuming and oleate-producing mutants. The primary cells were stabilized through multiple sub-culturing steps, leading to the isolation of a stable mutant that was selected for further enhancement. This mutant was subjected to ethyl methanesulfonate (EMS) mutagenesis and enrichment in lipid production media containing membrane fluidizing chemicals.

This process aimed to increase genetic diversity and select mutants with enhanced oleate levels. After multiple rounds of selection and enrichment, the mutants were isolated and screened, leading to the identification of another clone isolate which demonstrated a substantial increase in oleate content and lipid titer compared to its parental mutant. Its performance was validated under high cell density fermentation conditions. Additional rounds of classical strain improvement were conducted by exposing the strain to UV mutagenesis using a Stratalinker UV crosslinker (4000 W/cm^2 @254 nm for 10–30 s in order to achieve a kill rate of 90–95% versus untreated control plating; Stratagene, La Jolla, CA, USA). The mutated population was grown in lipid production medium containing the

mTOR inhibitor AZD8055 to select for desirable mutants. Cells were fractionated using a density gradient such that the highest oil producers would fractionate higher in the gradient. Mutants derived from the most buoyant fractions were subsequently assessed in lipid production medium for glucose consumption and fatty acid profile. A stable clone from this population underwent additional rounds of classical strain improvement using 4-NQO induced mutagenesis, followed by selection in lipid production media with clomiphene and emulsified linoleic acid to enrich for mutants producing lower levels of linoleic acid.

This process involved multiple cycles of exposure and selection, leading to the emergence of a resistant population. Single colonies from this population were isolated and screened for glucose consumption and fatty acid profile, resulting in the identification and stabilization of a mutant clone exhibiting high oil productivity (140 g/L) and a level of oleic acid comprising >86% of total fatty acids (see Figure 3B). The triacylglycerol (TAG) analysis of the oil by LC–MS revealed that triolein is the main TAG species present in this oil, reaching 68% of total TAG. In summary, the development involved a multi-step process of classical strain improvement and mutagenesis, including the creation and optimization of intermediate strains, followed by rigorous selection and stabilization strategies to enhance oleic acid content and overall strain performance.

3.3. Fermentation Trials at Scale

Fermentation trials were carried out with the high-oleic strain at scales ranging from 1 to 4000 L. Using a fed batch process with pH ranging from 5–7 and 1–2 vvm aeration rate, we were able to achieve biomass titers in the range of 200 g/L on a dry cell weight basis comprising 70–80 g of oil per 100 g of biomass. Detailed results for the high oleic strain in 1 L, 20 and 4000 L vessels are provided in Table 1, and comparison plots showing the dry cell weight (DCW) and volumetric oil production for the base strain versus the classically improved high oleic strain at the 1L scale are shown in Figure 2. For high oleic algae oil production, 1 L and 20 L runs were carried out using a final nitrogen concentration of 320 mM. The 4000 L runs utilized a 170 mM process. The differences reflect the lower oxygen transfer rate (OTR) achievable in the larger vessel, necessitating a process that would put fewer cells in place to stay within the capabilities of the fermentor. The differences at the 20 L, 1 L, and 4000 L scales also reflect differences in the vessels' configuration. Due to a relatively high z-axis fixed impeller position, the 1 L and 4000 L fermentors required higher initial operating volumes and hence earlier draws, reducing their final oil and DCW titers.

4. Discussion

Numerous unicellular microorganisms belonging to microalgal, bacterial, and fungal lineages possess the capacity to produce lipids utilizing various carbon sources [2,10]. Comprehensive literature research has been conducted, and a detailed dataset is available as Supplementary Material (Table S1). Among bacteria, the most documented species include: *Arthrobacter* sp., *Acinetobacter calcoacetinus*, *Rhodococcus opacus*, *Rhodococcus jostii*, *Rhodococcus rhodochrous*, *Gordonia* sp., and *Rhodococcus opacus.* These have high oil contents (ranging from 4–70%), but at low lipid titers (ca. 5 g/L) [11–13]. The production of lipids via fungal fermentation has been investigated [11–16]. The most promising fungal species, such as *Mortierella isabellina*, can yield biomass containing up to 80% oil with robust productivity [12,14,15]. An important number of yeasts have been investigated [11–15,17–24], and the most extensively studied strain remains *Yarrowia lipolytica*, capable of accumulating up to 87% lipids and achieving lipid titers nearing 85 g of oil per liter [17]. The vast array of microalgae and Thraustochytrids have been studied [1,11,12,14,25], and certain *Chlorella* species can yield biomass containing > 70% lipids and achieve lipid titers exceeding 50 g of oil per liter [1,14].

Among the over 150 microorganism species studied for their lipid-producing capabilities, very few are utilized on an industrial scale due to their low productivity, which has rendered commercial exploitation challenging (see Supplementary Table S1). The yeast

Yarrowia lipolytica stands out as one of the most utilized platforms for the development of industrial strains [11–14,17,18,20,22,24]. Recently, Wang and colleagues developed a strain capable of producing high-oleic acid oil through genetic engineering [21] with high oleic acid content and oil titers compared to those previously obtained with *Rhodotorula glutinis* [20], *Mortierella alpina* [16], *Trichosporon capitatum* (19), and *Yarrowia lipolytica* [24]. As depicted in Figure 4, the best results achieved by Wang and colleagues with *Yarrowia lipolytica* remain below those obtained in the present study: 145 g of oil per L of media with *P. moriformis* versus 56 g of oil per L for *Yarrowia lipolytica* [22]. Moreover, the level of oleic acid obtained is higher in this study (>86 versus 83% of total fatty acids) without the utilization of genetic engineering tools.

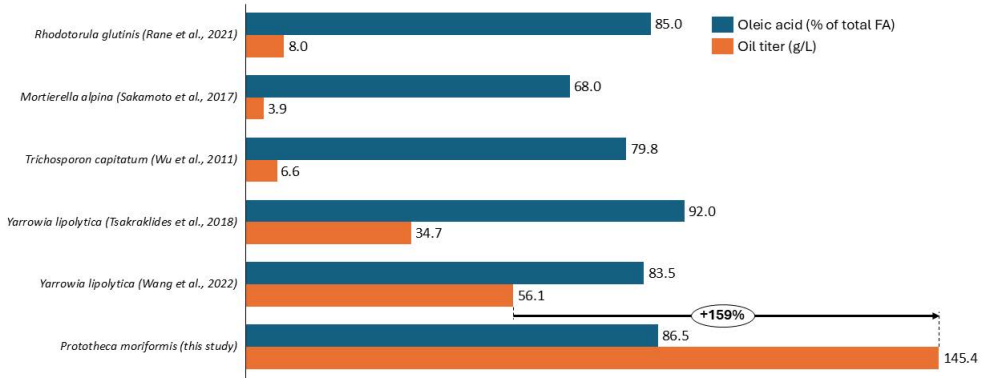

Figure 4. Comparison of the oleic acid in % of total fatty acids (blue bars) and oil titer in g per L of media (orange bars) in high-oleic acid oils produced by microbial strains reported in the literature [16,19,21,22,24] as well as in the present study.

High oleic oils are gaining prominence in food and industrial sectors due to their distinctive chemical properties, particularly their oxidative stability and health benefits [26]. Apart from food products, high oleic oils are extensively used in non-food applications, including lubricants and the personal and home care industries.

There are various types of high oleic oils, each offering distinct properties [26–30]. Olive oil, one of the most well-known, is a staple in the Mediterranean diet. It contains 55–83% oleic acid and is recognized for its cardiovascular benefits. Olive oil is highly suitable for cooking and other food applications due to its stability, and its minor components, such as polyphenols and tocopherols, further enhance its health benefits and oxidative stability. Similarly, avocado oil, with 60–70% oleic acid, is known for its excellent oxidative stability and valuable health-promoting compounds like phytosterols and tocopherols. It is used in culinary applications and is highly regarded for its moisturizing properties, making it a popular ingredient in personal care products. Macadamia oil, which contains around 60–80% oleic acid, is rich in oleic and palmitoleic acid, known for its anti-inflammatory and skin-nourishing properties. This makes macadamia oil a favored choice in the cosmetic industry and food production. High oleic sunflower oil, developed through plant breeding, contains more than 80% oleic acid and has gained traction for frying and baking due to its superior stability compared to conventional sunflower oil. It is increasingly used in cosmetics and personal care products for its light texture and non-greasy feel. High oleic soybean oil, developed through genetic modification, has more than 70% oleic acid and offers a stable and healthier alternative to traditional soybean oil, which is higher in polyunsaturated fats. Its stability makes it particularly popular in processed food production, especially for frying and baking.

The growing demand for high oleic oils is driven by changing consumer preferences and regulatory changes [26,31]. The FDA's ban on partially hydrogenated oils (PHOs), the primary source of artificial trans fats, has forced manufacturers to seek alternatives that provide similar stability without the health risks. High oleic oils, with their low levels of polyunsaturated fatty acids, have emerged as the ideal solution, offering the oxidative

stability required without the need for hydrogenation. Consumer awareness of the health risks associated with saturated and trans fats has further fueled the demand for oils rich in monounsaturated fats like oleic acid. High oleic oils are seen as healthier alternatives due to their ability to reduce LDL cholesterol while maintaining or improving HDL cholesterol levels. Their oxidative stability also makes them ideal for frying, baking, and shelf-stable products. The food industry has embraced high oleic oils in products that require long shelf lives and resistance to oxidation, such as snack foods, baked goods, and ready-to-eat meals.

Beyond food applications, high oleic oils have become popular in technical industries, including bio-based lubricants, coatings, and personal care products [32]. Their stability and sustainability make them appealing alternatives to traditional oils. The personal care industry, in particular, values these oils for their moisturizing and anti-inflammatory properties, which make them ideal for skin care and cosmetic formulations. In personal care, high oleic oils, such as avocado, macadamia, and olive oil, are favored in skincare and haircare products. Their emollient properties make them ideal for moisturizers, lotions, and conditioners. These oils provide deep hydration without clogging pores, making them suitable for all skin types, including sensitive and acne-prone skin. Their anti-inflammatory and antioxidant properties promote healthier, more resilient skin. High oleic oils are also used in home care products, where their biodegradability and non-toxic nature make them ideal for environmentally friendly cleaning products and surface treatments. Oleic acid and alcohol derivatives act as solvents and carriers for active ingredients in detergents, soaps, and polishes. In technical industries, high oleic oils are used in bio-based lubricants and hydraulic fluids, offering a renewable alternative to petroleum-based products. Their high stability at elevated temperatures makes them suitable for industrial machinery, automotive applications, and as additives in rubber and asphalt.

High oleic oil produced through the fermentation of *P. moriformis* offers significant advantages, particularly in sustainability and environmental safety. The fermentation process is performed in controlled environments, reducing the risk of contamination by environmental contaminants. This controlled production ensures a purer product while minimizing the ecological footprint, as heterotrophic algae can be grown in bioreactors, requiring less water and land use. The fermentation process allowsthe precise production of oils with consistent oleic acid content above the level of conventional high-oleic oil, ensuring quality and stability across batches. From a sustainability standpoint, oil production by fermentation can decouple the oil supply from traditional agricultural dependencies, making it more resilient to fluctuations caused by climate conditions, pests, or crop diseases. This independence from traditional farming provides a stable and scalable source of high oleic oil, particularly valuable as global demand grows. As sustainability becomes increasingly critical (lower green-house gas emissions, eutrophication, land efficiency/yield, water management), fermentation offers a highly scalable alternative that supports supply chain resilience while delivering a high-quality oil with superior level of monounsaturated fatty acids.

5. Conclusions

With oleic acid > 86% of total fatty acids, the oil developed in this study could drive competitive advantages in the realm of sustainable lipid production. By focusing on enhancing the fatty acid composition of microalgae oils, this approach offers a promising pathway to meet evolving demands across a variety of applications, including food, nutrition, personal care, and materials. The exceptionally high levels of oil production and oleic acid content achieved in this microalgae strain, reaching higher levels than those found in conventional oils like high-oleic sunflower, canola, and soybean oils, underscores the transformative potential of this technology. Importantly, the utilization of classical strain optimization techniques circumvents the need for genetic engineering, thereby addressing consumer preferences for non-genetically derived products while simplifying regulatory pathways and reducing developmental costs. Moreover, by leveraging native microorganisms and optimizing their lipid production capabilities, this approach con-

tributes significantly to mitigating environmental concerns associated with conventional oil production by increasing yields and land use efficiencies beyond those of conventional seed oils. The enhanced purity and sustainability of the oils developed through this methodology underscore their potential to redefine industry standards, offering not only superior nutritional profiles but also contributing to lower environmental impacts and improved overall product quality across diverse industrial segments.

6. Patents

The data presented in this paper are described in the patent US011873405B entitled "High-oleic oil compositions and uses thereof".

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10110566/s1. The comprehensive literature research data related to oil production in different microorganisms is available as Supplementary Material (Table S1).

Author Contributions: L.P., K.W., T.P., J.P., P.D. and S.F. developed the algae strain. D.A., B.D., L.E., N.R. and G.A. performed the fermentation trials. J.W. and G.E. performed the extraction and refining of the algae oil samples. M.C., V.B. and R.M. conducted all the analytical method development and quantification of fatty acids and F.D., L.P., W.R. and S.F. determined the target composition from literature and wrote the manuscript draft. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: All authors are team members of Checkerspot, Inc., headquartered in Alameda, CA, USA and declare no conflicts of interest.

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