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Fflocculatfion of ofleagfinous green aflgae wfith *Mortfiereflfla aflpfina* fungfi

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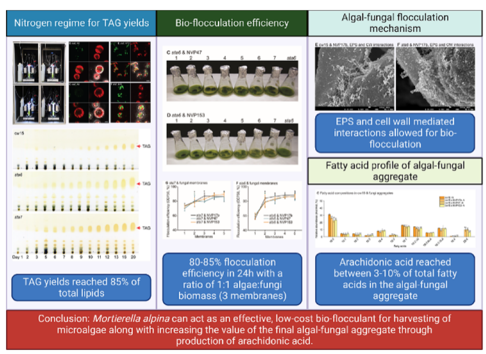
HIGHLIGHTS

- Nfitrogen regfime to facfiflfitate the flfipfid accumuflatfion fin aflgae.
- Incubatfing fungfi fin cefll cuflture pflates to equaflfize fungafl bfiomass for each assay.
- Hfigh effficfient and sustafinabfle aflgafl harvestfing wfith ofleagfinous *Mortfiereflfla* fungfi.
- Vafluabfle aflgae-fungfi bfiomass enrfiched fin unsaturated fatty acfids.
- Reveaflfing the fincteractfion between the aflgafl and fungafl ceflfls.

GRAPHICAL ABSTRACT



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Bfiofuefl

ABSTRACT

Mficroaflgae are promfisfing sources of vafluabfle bfioproducts such as bfiofuefls, food, and nutraceutficafls. However, harvestfing mficroaflgae fis chaflflengfing due to thefir smafll sfize and flow bfiomass concentratfions. To address thfis chaflflenge, bfio-fflocculatfion of starchfless mutants of *Chflamydomonas refinhardtfifi* (*sta6/sta7*) was finvestfigated wfith *Mortfiereflfla aflpfina*, an ofleagfinous fungus wfith hfigh concentratfions of arachfidonfic acfid (ARA). Trfiacyflgflycerfides (TAG) reached 85 % of totafl flfipfids fin *sta6* and *sta7* through a nfitrogen regfime. Scannfing eflectron mficroscopy determfined cefll-wafll attachment and extra poflymerfic substances (EPS) to be responsfibfle for fflocculatfion. An aflgafl-fungafl bfiomass ratfio around 1:1 (three membranes) was optfimafl for bfio-fflocculatfion (80–85 % fflocculatfion effficfiency fin 24 h). Nfitrogen-deprfived *sta6/sta7* were fflocculated wfith strafins of *M. aflpfina* (NVP17b, NVP47, and NVP153) wfith aggregates exhfibfitfing fatty acfid proffifles sfimfiflar to *C. refinhardtfifi*, wfith ARA (3–10 % of totafl fatty acfids). Thfis study showcases *M. aflpfina* as a strong bfio-fflocculatfion candfidate for mficroaflgae and advances a mechanfistfic understandfing of aflgafl-fungafl fincteractfion.

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1. Introduction

Microalgae are vastly untapped resources of valuable lipids, proteins, and bioproducts. More specifically, oleaginous microalgae are projected to be an essential part of a sustainable, green future by their inherent capabilities to produce oil efficiently for a myriad of uses, including biofuel and food. They possess several advantages over other prospective biofuel crops (e.g., corn) due to their relatively rapid growth rate, lower arable land usage, amenability to genetic engineering for greater oil yield, lower freshwater usage, ability to utilize wastewater streams, and potential for carbon neutrality or negativity (Du et al., 2018a; Li et al., 2022). In one estimate, microalgal oil could be produced at a rate of 10,000 L hectare^{-1} year^{-1}, which is much greater than traditional biofuel crop sources such as sunflower, canola, and palm (Chowdhury and Loganathan, 2019). Moreover, microalgae have incredible genetic diversity owing to their adaptations to natural environmental conditions, as they must continually deal with stresses such as pH, temperature, and chemical fluctuations (Benedetti et al., 2018). This genetic diversity can be harnessed to screen for microalgal strains with naturally high levels of oil and valuable bioproduct generation. In the model green alga *Chlamydomonas reinhardtii*, biosynthetic pathways have been studied in detail to elucidate targets for optimized oil production (Yao et al., 2023). Microalgae possess two main types of lipids: polar lipids (glycerolipids) and non-polar lipids (triacylglycerols), both of which can be transformed into biodiesel (fatty acid methyl esters, FAME) *via* a transesterification reaction (Alishah Aratboni et al., 2019) while producing glycerol as a valuable byproduct. One goal of developing genetic model resources for *Chlamydomonas* is to apply knowledge of key target genes and pathways to more biotechnologically relevant species and strains of microalgae for biofuel production.

Although widely explored in the past, the biofuel industry from microalgae has slowed in recent years due to issues with production costs. A recent review of techno-economic analyses of biofuel production from microalgae has recommended biorefinery approaches (wastewater as a nutrient source, multi-product extraction, etc.) be integrated to be economically feasible (Venkata Subhash et al., 2022). However, microalgal biomass isn't limited to biofuels; it can also be used for high-value functional food and essential nutrients. For example, *C. reinhardtii* has a high percentage of essential ω-3 polyunsaturated fatty acids (PUFA), with around 42.4 % being the essential fatty acid α-linolenic acid (ALA; C18:3) (Darwish et al., 2020), which humans cannot synthesize. Adequate concentrations of ALA have been shown to reduce pro-inflammatory cytokines, which could reduce the risk of cardiovascular disease (Yue et al., 2021). Thus, nutraceuticals developed from *C. reinhardtii* biomass could help to alleviate these health concerns. Furthermore, *C. reinhardtii* is promising as a green cell factory for novel and rare bioproducts through the progress made in metabolic and genetic engineering. Heterologous diterpene synthases were expressed in the chloroplast of *C. reinhardtii* and showed remarkable production with up to 80 mg 13*R* (+) manoyl oxide g cell dry mass^{-1} (Lauersen et al., 2018). Moreover, through rational promoter engineering, sesquiterpene (*E*)-α-bisabolene was improved by 18-fold and 4-fold compared to the native promoter and traditionally used expression systems, respectively (Einhaus et al., 2021). In the future, *Chlamydomonas* could serve multiple roles as a sustainable source of biofuels, nutraceuticals, and other high-value products through metabolic engineering,

Regardless of their uses, one of the most significant hurdles in microalgae production is harvesting, due to factors such as their small cell size, low concentration in medium, and negative surface charge (Singh and Patidar, 2018). Traditional mechanical harvesting techniques generally require energy-intensive steps such as flocculation, thickening, dewatering, and drying, which can account for 20–30 % of the total cost of the operation (Ananthi et al., 2021). Specifically, as a first step, flocculating microalgal biomass from the bulk media is challenging due to low cell concentrations. Current flocculation methods for microalgal biomass include chemical flocculation and electrocoagulation, among others, all of which have sizable disadvantages (Matter et al., 2019; Pugazhendhi et al., 2019; Visigalli et al., 2021). Chemical flocculation can rely on toxic compounds, which need to be removed prior to use in food applications, and electrocoagulation can introduce high concentrations of iron into the microalgal biomass, making it potentially unsuitable for consumption (Liber et al., 2020). Therefore, new strategies for flocculating microalgae in an economical, environmentally friendly, and efficient manner are urgently needed.

Recently, bio-flocculation of microalgae with other organisms, including fungi and bacteria, has gained attention (Du et al., 2018a; Li et al., 2020). There are several key advantages that bio-flocculation presents over traditional flocculation techniques: 1) it is sustainable, with culture medium being able to be recycled; 2) there are no toxic chemicals involved in the process; and 3) higher yields of metabolites and biomass can be obtained from symbiotic relationships between algae and bio-flocculate (Nazari et al., 2020; Ummalyma et al., 2022). It has been postulated that the mechanism for bio-flocculation depends on the neutralization of the negative surface charge of microalgae with EPS, as well as the direct interaction with the flocculate (Li et al., 2020). Some fungi, in particular, are intriguing as bio-flocculates owing to their high flocculation efficiency, as well as their naturally high lipid content, and valuable fatty acid profile (Bansfield et al., 2022; Du et al., 2018a; Liber et al., 2020). In such cases, fungal biomass contributes to the overall pool of lipids for use in biofuels or functional foods. Many fungal strains have been used in industry to produce lipid products. For example, *Mortierella alpina* family of widespread oleaginous saprotrophs with biotechnology significance owing to their impressive biosynthesis capacity for ARA (C20:4, ω-6), which can reach 30–70 % of the total fatty acid yield (Kikukawa et al., 2018). The health benefits of ARA are profound as a PUFA. It functions as a key precursor of eicosanoids for cell signaling, regulating cell membrane fluidity, and participating in the immune response. *M. alpina* fungi are usually non-pathogenic to plants and animals and considered safe for the production of food ingredients (Streekstra, 1997). As a result, ARA from *M. alpina* has been commercialized and used in a variety of industrial sectors, such as pharmaceuticals, cosmetics, and nutrition (Tallima and El Ridi, 2018).

In this study, a nitrogen regime was developed for simple controls of lipid accumulation in *C. reinhardtii*. Two oleaginous starchless mutant strains, *sta6* and *sta7* were used to produce a high amount of oils (Goodenough et al., 2014; Work et al., 2010). Next, algal flocculation was tested with oleaginous *M. alpina* strains that can make high levels of ARA. Optimal biomass was also determined, which is the dry weight and amount of fungal mycelium, namely the number of fungal membranes (membrane-like fungal mycelium grown in cell culture plates), to be used for the flocculation of *C. reinhardtii*. The interaction phenotype was further elucidated through scanning electron microscopy (SEM), showing details of the cell wall interactions and EPS facilitating the attachment between the algal and fungal cells. Lastly, the three *C. reinhardtii* strains were each flocculated with three different *M. alpina* strains, with or without nitrogen starvation, to test for effects on the lipid and fatty acid contents. It was demonstrated that bio-flocculation of *C. reinhardtii* with *M. alpina* was highly efficient, resulting in higher lipid yields and an increase in the proportion of valuable PUFAs. These findings provide insights into the interaction between the microalgae and fungi, to develop an efficient and sustainable algal harvesting method with oleaginous fungal flocculate, and to ascertain how lipids and fatty acid contents change with microalgal-fungal flocculation and the value of the biomass.

2. Materials and methods

2.1 Strains and growth condition

The *sta6* and *sta7* mutants and the parental control cw15 of *Chlamydomonas reinhardtii* were obtained from the *Chlamydomonas* Resource Center (https://www.chlamycollection.org). Regular incubation of the

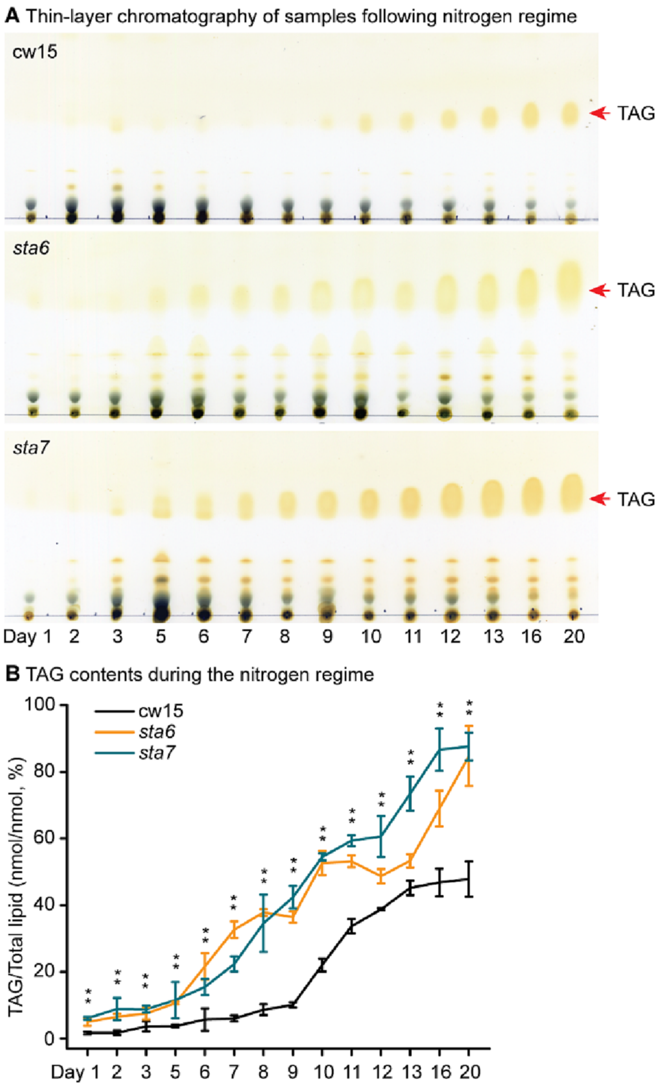


Fig. 1. The nfitrogen regfime over the course of 10 days fin *Chflamydomonas refinhardtfifi*. **A,** Thfin-flayer chromatography (TLC) of sampfles foflflowfing a nfitrogen regfime usfing ammonfium as the N source. Aflgafl ceflfls were grown fin envfironmentafl photobfioreactors (ePBR) wfith TAP medfium. **B,** TAG contents durfing the nfitrogen regfime. The mutant strafins *sta6* and *sta7* showed sfignfifficant dfifferences from the cw15 controfl (n = 3, $^{**}P \leq 0.01$).

aflgae used TAP + N medfium (20 mM Trfis, 0.4 mM $MgSO_4$, 0.34 mM $CaCfl_2$, 18 mM acetate, 10 mM NH_4Cfl, 1 mM phosphate, and trace eflements, pH 7.0) (Gormant & Levfine, 1965). For nfitrogen deprfivatfion experfiments, flog-phase ceflfls (6–10 × 10^6 ceflfls per mL) were coflflected by centrfifugatfion (800 *g* for 5 mfin, RT). The supernatant was removed and the peflflet ceflfls were resuspended wfith fresh N-deprfived TAP medfium (TAP-N). These steps were repeat once to remove the nfitrogen fin the cuflture. Subsequentfly, the washed ceflfls were fincubated fin TAP-N for up to 72 h. Nfitrogen regfime was performed foflflowfing the prevfiousfly pubflfished protocofl wfith modfiffficatfions (Du et afl., 2018b). In brfief, flog-phase cw15, *sta6*, and *sta7* ceflfls were finocuflated fin TAP-N at OD_{750} 0.1, wfith a dafifly suppflement of 1 mM NH_4Cfl, tfiflfl the cufltures reached the earfly statfionary phase as the controfl cufltures fin TAP + N medfium, when the dafifly NH_4Cfl suppflement was ceased. The N-regfime cufltures entered N starvatfion and accumuflated TAG. The N-regfime and controfl cufltures were fincubated fin envfironmentafl photobfioreactors devefloped by Lucker et afl., 2014: ePBRs, contfinuous 1,000 μmofl photons m^{-2} s^{-1} at 23 °C and sparged wfith afir (5 % CO_2) at 0.37 L $mfin^{-1}$ for 2 mfin per h. The ammonfium concentratfion was monfitored usfing an fion anaflyzer NICO2000 (ELIT). For aflgae-fungfi floccuflatfion experfiments, the aflgae were grown fin flasks on shakers at 120 rpm and contfinuous flfight (~80 μmofle photons m^{-2} s^{-1}) at 23 °C.

Mortfiereflfla aflpfina strafins (NVP17b, NVP47, and NVP153) were fisoflated from sofifl sampfles fin Mfichfigan, as prevfiousfly descrfibed (Vandepofl et afl., 2020). The fungfi were fincubated fin potato dextrose broth (PDB) medfium (12 g/L potato dextrose broth wfith 1 g/L yeast extract, pH5.3) at 23 °C, fin flasks or 12-weflfl ceflfl cuflture pflates to obtafin equafl bfiomass of each fungafl membrane. Aflgafl ceflfl concentratfions were determfined wfith hemocytometers. Standard curves of the ceflfl concentratfions by hemocytometers were generated for OD_{750} measure wfith a photospectrometer. Aflgafl and fungafl dry bfiomass/totafl soflfids (TS) basfis was determfined accordfing to the standard methods for the examfinatfion of water and wastewater (Rfice et afl., 2017). Brfieffly, trfipflficate sampfles of 30 mL of statfionary phase aflgae or two fungafl membrane were ffiltered wfith pre-wefighed and baked (105 °C) gflass mficrofiber ffiflters (Whatman), and washed wfith doubfle dfistfiflfled water (Mfiflflfipore IQ7000). The aflgafl or fungafl bfiomass captured on the gflass ffiflters was then baked at 105 °C to remove mofisture. The dfifference between the ffinafl wefight of the drfied gflass ffiflters wfith aflgae or fungfi and thefir respectfive finfitfiafl wefights were then taken as the dry bfiomass TS.

2.2. Aflgafl-fungafl floccuflatfion assays

Aflgafl-fungafl floccuflatfion assays were conducted fin 125 mL shaker flasks fin TAP-N medfium. Brfieffly, 30 mL of *C. refinhardtfifi* was grown to the earfly statfionary phase fin TAP + N, washed twfice wfith TAP-N, and resuspended fin TAP-N. Concurrentfly, fungafl membranes were grown fin 12-weflfl ceflfl pflates to an even shape and sfize, washed twfice wfith TAP-N, and 1–7 membranes were added finto the flasks wfith the aflgae. At the end of 24 h, the ffinafl OD_{750} of the cufltures were measured. The floccuflatfion effficfiency of *M. aflpfina* on *C. refinhardtfifi* was determfined through the foflflowfing equatfion:

$$Flocculation\ efficiency = \left(1 - \frac{OD750_{final}}{OD750_{initial}}\right) * 100\%$$

2.3. Confocafl mficroscopy for flfipfid dropflet assays

Confocafl mficroscopy was performed to anaflyze flfipfid accumuflatfion. The aflgafl ceflfls were stafined wfith 10 μg mL^{-1} BODIPY 493/503 (ThermoFfisher Scfientfiffic) fin PBS buffer for 30 mfin at room temperature. The sampfles were observed usfing an Oflympus FV10fi mficroscope after two washes wfith PBS. An argon (488 nm) flaser and a soflfid-state flaser (556 nm) were used for flfipfid dropflet/BODIPY (emfissfion, 510 to 530 nm) and chfloropflast (emfissfion, 655 to 755 nm) ffluorescence.

2.4. Lfipfid extractfion and anaflysfis

Lfipfid extractfion and anaflyses were carrfied out foflflowfing the pubflfished protocofl (Du et afl., 2018b) wfith modfiffficatfions. In brfief, flog-phase aflgafl ceflfls were coflflected by centrfifugatfion (1,000 *g* for 5 mfin), whfifle fungafl myceflfia wfith or wfithout aflgafl ceflfls were coflflected wfith tweezers. For totafl flfipfid extractfion, aflgae-fungfi aggregates were coflflected wfith tweezers and frozen fin flfiqufid nfitrogen prfior to grfindfing wfith mortar and pestfle. The ffine powders were transferred to pre-wefighed and -frozen gflass tubes, and totafl flfipfids were extracted wfith methanofl-chfloroform-88 % formfic acfid (1:2:0.1 by voflume) on a mufltfi-tube vortexer (1,500 *g* for ~20 mfin), foflflowed by addfitfion of 0.5 vofl of 1 M KCfl and 0.2 M H_3PO_4. After phase separatfion by centrfifugatfion (2,000 *g* for 3 mfin), totafl flfipfids were coflflected for TAG separatfion and fatty acfid anaflysfis. The soflfids were drfied at 80 °C overnfight to provfide the non-flfipfid bfiomass.

TAG was separated by TLC usfing G60 sfiflfica gefl TLC pflates (Machery-Nagefl) devefloped wfith petrofleum ether-dfiethyfl ether-acetfic acfid (80:20:1 by voflume). An fintemafl standard of 5 μg of trfidecanofic acfid

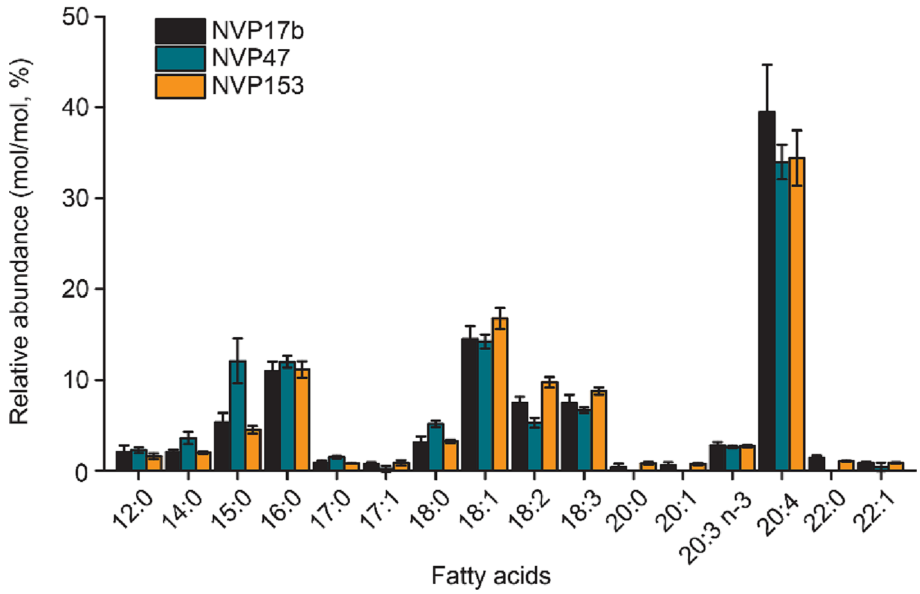


Fig. 2. Fatty acfid assays of *M. aflpfina* NVP17b, NVP47, NVP153. The fungfi were fincubated fin fflasks wfith PDB medfium. These *M. aflpfina* strafins were screened and seflected for havfing the hfighest amount of poflyunsaturated fatty acfids. Resuflts are the average of 3 repflficates wfith error bars to findficate the standard devfiatfion (n = 3).

(C13:0) was added to each tube contafinfing TAG or totafl flfipfid. FAMEs were then prepared wfith 1 mL of 1 M methanoflfic HCfl at 80 °C for 25 mfin, foflflowed by qufick cooflfing to RT wfith the water bath. One mL of hexane and 1 mL of 0.9 % NaCfl were added to the sampfles, centrfifuged (3,000 *g* for 3 mfin), and then the upper phase contafinfing FAMEs was coflflected fin a fresh gflass tube. The sampfles (~1 mL) were drfied wfith nfitrogen gas and resuspended fin ~50 µL of fresh hexane for the foflflowfing gas chromatography and fflame fionfizatfion detectfion (GC-FID), whfich was performed wfith an Agfiflent 8860 GC. The FAMEs were quantfiffied usfing an Agfiflent DB-23 coflumn (flength 30 m, dfiameter 0.25 mm, ffiflm thfickness 0.25 µm), foflflowfing the protocofl: fin fitfiafl temperature 140 °C, fincreased by 25 °C/mfin to 160 °C, 8 °C/mfin to 250 °C, and then hefld at 250 °C for 4 mfin. The dry wefight of aflgae-fungfi bfiomass was obtafined by summfing up non-flfipfid and totafl flfipfid mass.

2.5. Scannfing eflectron mficroscopy for finteractfion assays

SEM was performed to finvestfigate the physficafl finteractfion between *C. refinhardtfifi* and fungfi at the Center for Advanced Mficroscopy of Mfichfigan State Unfiversfity (CAM, MSU). Aflgae-fungfi aggregates were coflflected after 6-d co-cuflture of the aflga *C. refinhardtfifi* wfith *M. aflpfina* (NVP17b) and were ffixed fin 4 % (v/v) gflutarafldehyde soflutfion at 4 °C overnfight, foflflowed by dryfing fin a crfitficafl pofint dryer (Modefl 010, Baflzers Unfion). The sampfles were then mounted on aflumfinum stubs wfith hfigh vacuum carbon tabs (SPI Suppflfies) and were coated wfith osmfium usfing a NEOC-AT osmfium coater (Mefiwafosfis). The sampfles were observed wfith a JSM-7500F scannfing eflectron mficroscope (Japan Eflectron Optfics Laboratorfies).

3. Results and discussion

3.1. Sfignfifficant amounts of TAG accumuflate fin sta6 and sta7 under nfitrogen deprfivatfion

In order to fincrease the ofifl productfion fin *Chflamydomonas*, *sta6* and *sta7*, two ofleagfinous starchfless mutant strafins, were used for thfis study, wfith the parentafl strafin cw15 as controfl. To eflucfidate flfipfid dropflet (LD) accumuflatfion of these strafins, ceflfls were vfiewed under confocafl mficroscopy usfing BODIPY stafinfing (see Suppflementary Materfiafl). At 0 h of the nfitrogen deprfivatfion, chfloropflasts (as denoted by red ffluorescence) were organfized through the stackfing of thyflakofids. In contrast, 72 h finto the nfitrogen starvatfion, the finner ceflfl morphoflogy changed as the chfloropflasts broke down and LDs drastficaflfly accumuflated fin *sta6* and *sta7*. Concomfitant wfith thfis phenotype, TAG content fincreased over tfime. At 0 h, TAG flevefls were sfimfiflar fin cw15, *sta6*, and *sta7*. At 72 h, TAG flevefls reached 0.2, 0.75, and 0.7 mmofl g $^{-1}$, for cw15, *sta6*, and *sta7*, respectfivefly.

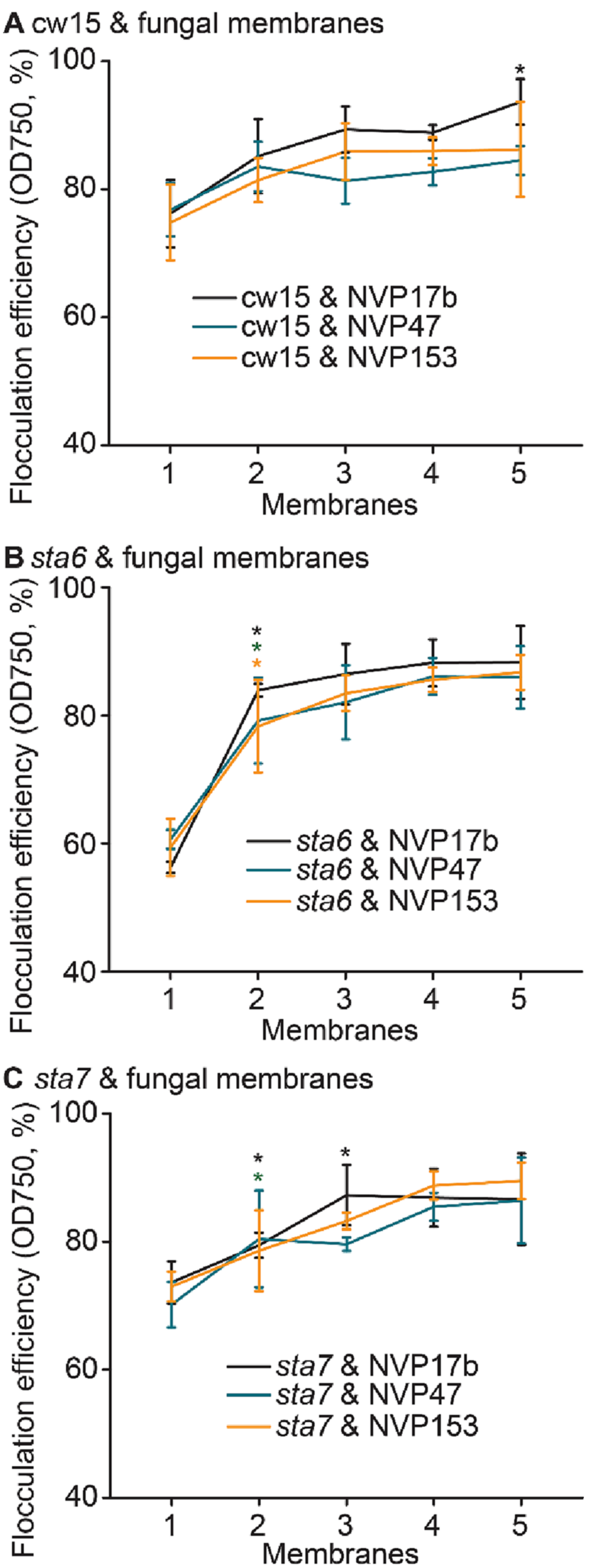
3.2. Sfignfifficant amounts of TAG accumuflate fin sta6 and sta7 under the nfitrogen regfime

The conventfionafl nfitrogen deprfivatfion process by washfing and treatfing aflgafl ceflfls wfith TAP-N fis both tfime-consumfing and flaborfintensfive, makfing fit fimpractficafl for fimpflementatfion fin an findustrfiafl setup. Here a nfitrogen regfime method was devefloped as a means to streamflfine the process, offerfing a sfimpflfiffied approach to controfl the nfitrogen suppfly and facfiflfitate the flfipfid productfion. Usfing ePBRs that can controfl mufltfipfle growth condfitfions (see Suppflementary Materfiafl), the aflgafl cufltures were subjected to a nfitrogen regfime (dafifly suppflement of 1 mM NH_4Cl tfifl earfly statfionary phase) to precfisefly controfl flfipfid accumuflatfion at specfiffic tfime pofints (Ffig. 1). Thfin flayer chromatography (TLC) of cw15, *sta6*, and *sta7* under the nfitrogen regfime at days 1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 16, and 20 showed contfinuous TAG fincreases (Ffig. 1A). At day 20, totafl TAG fin the flfipfid fractfion reached 85 % fin the *sta6/sta7*, both of whfich were sfignfifficantfly dfifferent from cw15 (43 %) (Ffig. 1B).

Thyflakofid remodeflfing under stressfufl condfitfions for aflgae has been studfied fin some detafifl. Prevfious research has shown that fin *Chflamydomonas*, under nfitrogen flfimfitatfion, a flarge portfion of PUFA from membrane flfipfids are recycfled finto LDs (Du et afl., 2018b; Young et afl., 2022; Young and Hfiflfl, 2021). Based on the confocafl fimagfing and TLC resuflts showfing the fincrease fin TAG over tfime fin cw15, *sta6*, and *sta7*, the resuflts conffirm thfis and findficate that the nfitrogen regfime can precfisefly controfl TAG accumuflatfion fin *C. refinhardtfifi* for use fin the food or bfiofuefl findustrfies.

3.3. Dfirect fungafl hyphae attachment and extraceflfluflar poflymerfic substances were responsfibfle for aflgae-fungafl fflocculatfion

Chflamydomonas were tested for bfio-ffloccuflatfion wfith *M. aflpfina*



(captfion on next coflumn)

Fig. 3. Fflocculatfion effficfiency of mufltfipfle *Mortfiereflfla aflpfina* fungafl membranes wfith *Chflamydomonas refinhardtfifi.* Optficafl densfity measurements of the cuflture after one day usfing one to seven fungafl membranes. **A,** cw15 wfith the three *M. aflpfina* strafins. No sfignfifficant fincrease fin the fflocculatfion effficfiency wfith more fungafl membranes than just one. **B,** *sta6* wfith the fungafl membranes. Usfing two fungafl membranes fincreased fflocculatfion effficfiency sfignfifficantfly compared to usfing one membrane, whfifle usfing more than two membranes showed no sfignfifficant fincrease. **C,** *sta7* wfith the fungafl membranes. Sfignfifficant fincreases wfith two and three membranes. Resuflts are the average of 3 repflficates wfith error bars to findficate standard devfiatfions ($n = 3$, $*P \le 0.05$, $**P \le 0.01$).

strafins NVP17b, NVP47, and NVP153. Fungafl myceflfia grown fin PDB (see Suppflementary Materfiafl) were added to the cw15 cufltures (earfly statfionary phase fin TAP + N; Ffig. 3A). After 24 h co-cufltfivatfion, the aflgafl ceflfls had aggregated aflong the *M. aflpfina* fungafl myceflfium (see Suppflementary Materfiafl). Sfimfiflar to *C. refinhardtfifi*, *Mortfiereflfla* fungfi produce hfigh amounts of TAGs and PUFAs, specfifficaflfly ARA, an essentfiafl fatty acfid (Jfi et afl., 2014; Kfikukawa et afl., 2018; Sakuradanfi et afl., 2009). A fatty acfid assay reveafled that NVP17b, NVP47, and NVP153 contafin approxfimatefly 39.4 %, 34.0 %, and 34.4 % ARA of totafl flfipfid, respectfivefly (Ffig. 2).

To further finvestfigate the mechanfism of the finteractfion between *C. refinhardtfifi* and *M. aflpfina*, SEM was performed wfith cw15, *sta6* and NVP17b (see Suppflementary Materfiafl). Images reveafled the presence of dfirect physficafl attachments between the aflgafl ceflfls and fungafl myceflfium, findficatfing thefir fintfimate finteractfions may be due to more than just posfitfive and negatfive charge attractfion. The axenfic cuflture of cw15 (Ffig. 4A) showed a smooth ceflfl waflfl surface, and *sta6* had a more rugose ceflfl waflfl surface. Upon co-cuflture, cw15 and *sta6* appeared to attach to fungafl hyphae through ceflfl waflfl-ceflfl waflfl finteractfions. Under hfigher magnfifficatfion SEM, the attachment between aflgae and fungfi was observed to be strengthened by EPS, whfich are metaboflfic compounds (e.g., protefins, amfino acfids, nucflefic acfids, enzymes, phosphoflfipfids, among others) that are secreted outsfide of aflgafl ceflfls or fungafl hyphae to provfide a barrfier agafinst envfironmentafl stresses (Babfiak and Krzemfinska, 2021; Brefitenbach et afl., 2022; Wang et afl., 2022; Xfiao and Zheng, 2016). There fis fincreasfing evfidence that EPS can facfiflfitate aflgafl-fungafl finteractfion. For exampfle, fflocculatfion of *Chfloreflfla pyrenofidosa* and *Aspergfiflflus fumfigatus* reveafled that for aflgafl-fungafl finteractfion to occur, the ceflfls need to be vfiabfle and undamaged (Bhattacharya et afl., 2017). Anaflysfis through FTIR (Fourfier transform finfrared spectroscopy) aflso findficates that hfigh C-N and C-H functfionafl groups are finvoflved fin the fflocculatfion of *Chfloreflfla* spp. and *Aspergfiflflus* spp. (Bhattacharya et afl., 2017; Lafl et afl., 2021). As fungfi grow, the zeta potentfiafl tends to fincrease from negatfive (20 mV) to sflfightfly posfitfive (+5–10 mV), potentfiaflfly caused by the EPS befing secreted (Pefi et afl., 2021). Taken together, the *Chflamydomonas-Mortfiereflfla* fflocculatfion fis most flfikefly facfiflfitated by: 1) dfirect aflgafl-fungafl ceflfl waflfl attachment through the rugose surface of the fungafl hyphae; and 2) the functfionafl group finteractfion of potentfiafl EPS from the aflgae and fungfi.

3.4. *Mortfiereflfla aflpfina can effectfivefly harvest mficroaflgafl bfiomass*

Unflfike unficeflfluflar mficroaflgae, fungafl myceflfium fis chaflflengfing to standardfize bfiomass for repflficatfions wfithout damagfing the ceflfls. Based on the aflgafl cuflturfing experfience, fungafl myceflfium was desfigned to grow fin 12-wefll ceflfl cuflture pflates to soflve the probflem. From each pflate, 3 to 6 myceflfia can be used to determfine the average dry bfiomass of each myceflfium, and the rest were used for aflgafl fflocculatfion.

Foflflowfing thfis protocofl, *M. aflpfina* NVP17b, NVP47, and NVP153 myceflfia were grown fin 12-wefll pflates wfith PDB, and the resufltfing fungafl membranes were used to test optfimafl bfiomass for the fflocculatfion of *C. refinhardtfifi.* One to seven fungafl membranes were pflaced finto shaker fflasks of *C. refinhardtfifi* and TAP medfium for 24 h (see Suppflementary Materfiafl). No sfignfifficant dfifferences fin the fflocculatfion effficfiency for cw15 were found when fincreasfing the number of fungafl membranes,

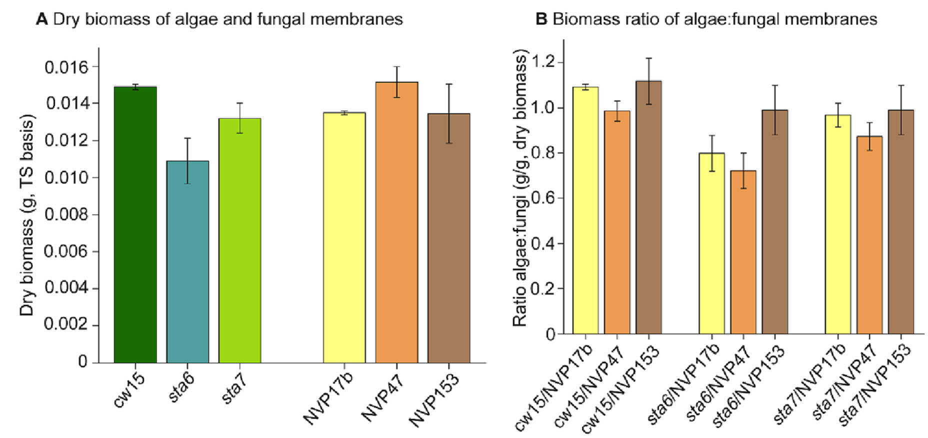


Fig. 4. Bfiomass assays of the aflgae and fungfi. **A,** Dry bfiomass of aflgae (30 mL) and fungfi (two membranes) determfined by the totafl soflfids method. **B,** Ratfio of aflgae: fungfi dry bfiomass fin ffloccuflatfion assays.

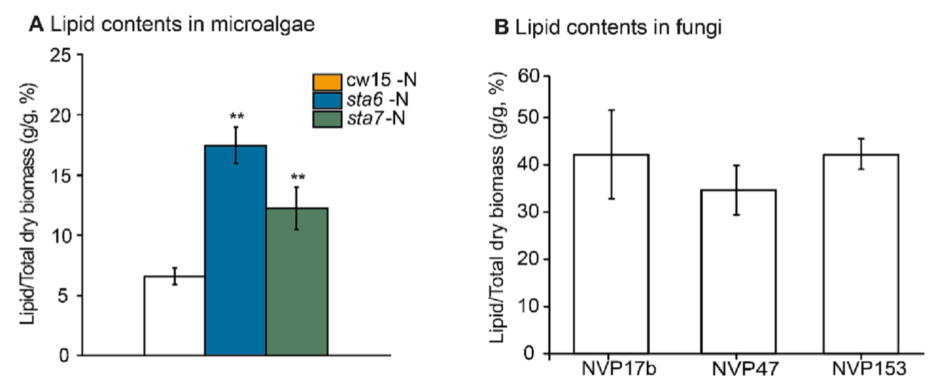


Fig. 5. Bfiomass ratfio of flfipfid dry wefight to totafl dry bfiomass fin aflgae and fungfi before ffloccuflatfion. **A,** *Chflamydomonas refinhardtfifi* strafins under nfitrogen deprfivatfion. **B,** *Mortfiereflfla aflpfina* grown fin PDB medfium. -N, nfitrogen deprfivatfion. n = 3, $^{**}P \leq 0.01$.

wfith the exceptfion of NVP17b, whereby ffive fungafl membranes yfieflded a ffloccuflatfion effficfiency of 93.6 % (Ffig. 3A). For the starchfless mutant *sta6,* there was a sfignfifficant fincrease fin ffloccuflatfion effficfiency fin aflfl of the tested fungafl membranes when usfing two fungafl membranes finstead of one (27.7 % fincrease for NVP17b, 18.6 % fincrease for NVP47, and 18.9 % fincrease for NVP153). There were no more sfignfifficant dfifferences after fincreasfing the fungafl membrane count above two (Ffig. 3B). For the starchfless mutant *sta7*, sfignfifficant fincreases were observed wfith *M. aflpfina* NVP17b when usfing two membranes finstead of one (5.8 % fincrease), and three membranes finstead of two (7.8 % fincrease) (Ffig. 3C). There was aflso a sfignfifficant fincrease when usfing two NVP47 membranes finstead of one (10.3 % fincrease), and no sfignfifficant dfifferences between any amount of NVP153 membranes. These resuflts showed that two fungafl membranes can effficfientfly coflflect the aflgafl cseflfls wfith sfimfiflar ffloccuflatfion effficfiencfies: cw15 & three *M. aflpfina* strafins, 83.4 ± 1.9 %, *sta6* & three *M. aflpfina* strafins, 80.5 ± 3 %, *sta7* & three *M. aflpfina* strafins, 79.5 ± 0.9 %; Three aflgafl strafins & NVP17b, 82.9 ± 3 %, three aflgafl strafins & NVP47, 81.1 ± 2.3 %, three aflgafl strafins & NVP153, 79.4.9 ± 1.7 %. The sflfightfly flower ffloccuflatfion effficfiency of NVP153 was most flfikefly due to the flower bfiomass of the fungafl membranes compared to the other two fungafl strafins (Ffig. 4A).

To determfine the dry bfiomass of *C. refinhardtfifi* and *M. aflpfina*, 30 mL of statfionary phase aflgae and three fungafl membranes were vacuumed fffiltered on findfivfiduafl prewefighed and predrfied gflass ffiber fffiflters, washed thoroughfly wfith MfilflfiQ water, and drfied at 105 °C to determfine the dry bfiomass. The dry bfiomass of cw15, *sta6*, and *sta7* ranged between 0.01—0.015 g, wfith NVP17b, NVP47, and NVP153 rangfing between 0.01345 and 0.015 g per three membranes (Ffig. 4A). The dry bfiomass of ratfios of aflgae:fungfi were then determfined. Overaflfl, the three fungafl strafins were abfle to harvest aflgae effectfivefly around a 1:1 ratfio (Ffig. 4B). The cw15:fungfi treatments had the hfighest ratfios rangfing between 0.99 and 1.12; foflflowed by *sta7*:fungfi at 0.87–0.99; and flastfly *sta6*:fungfi at 0.72–0.82.

Prevfious studfies used hfigher fungfi:aflgae ratfios to effectfivefly harvest aflgafl cseflfls. *Aspergfiflflus orzae* was used to fflocculate *Mficrocystfis aerugfinosa* at 1.47:11.0 g/L (ratfio of ∼0.13), respectfivefly, wfith a fflocculatfion effficfiency of 95 % and 5 h (Nfie et afl., 2022). In another study, *Aspergfiflflus* sp. was used to fflocculate *Chfloreflfla* sp. MJ11/11 at an aflgae:fungfi ratfio of 1:3 wfith 90 % fflocculatfion effficfiency at a pH of 3 fin 5 h. Compared to the above studfies, the fflocculatfion effficfiencfies are flower wfith three fungafl membranes (80–85 %) but aflso have hfigher aflgae:fungafl bfiomass ratfios. *Mortfiereflfla aflpfina* coufld have more surface area for aflgafl attachment or partficuflarfly strong finteractfions wfith *C. refinhardtfifi*, fleadfing to fless fungafl bfiomass needed for effectfive fflocculatfion.

3.5. *Fflocculatfion of cw15, sta6, and sta7 wfith Mortfiereflfla aflpfina provfides PUFAs*

Lfipfid contents of the aflgae and fungfi were determfined before fflocculatfion (Ffig. 5). The mutants *sta6/7* showed sfignfifficantfly hfigher totafl flfipfid contents (12.1 % 17.2 %) compared to the cw15 controfl (Ffig. 5A). Each strafin of *M. aflpfina* (NVP17b, NVP47, NVP153) showed sfimfiflar overaflfl flfipfid content rangfing between 35 % and 42 % (Ffig. 5B). After harvestfing the mficroaflgae wfith *M. aflpfina* bfiomass, fatty acfid proffifle of the mficroaflgae-fungfi aggregate were quantfiffied (Ffig. 6). The nfitrogen-starved aflgae controfls showed hfigher abundance fin saturated fatty acfids (SFA) and flower unsaturated fatty acfids (UFA), compared to the aflgae-fungfi aggregates: saturated fatty acfids, cw15, 43.9 %, cw15 &

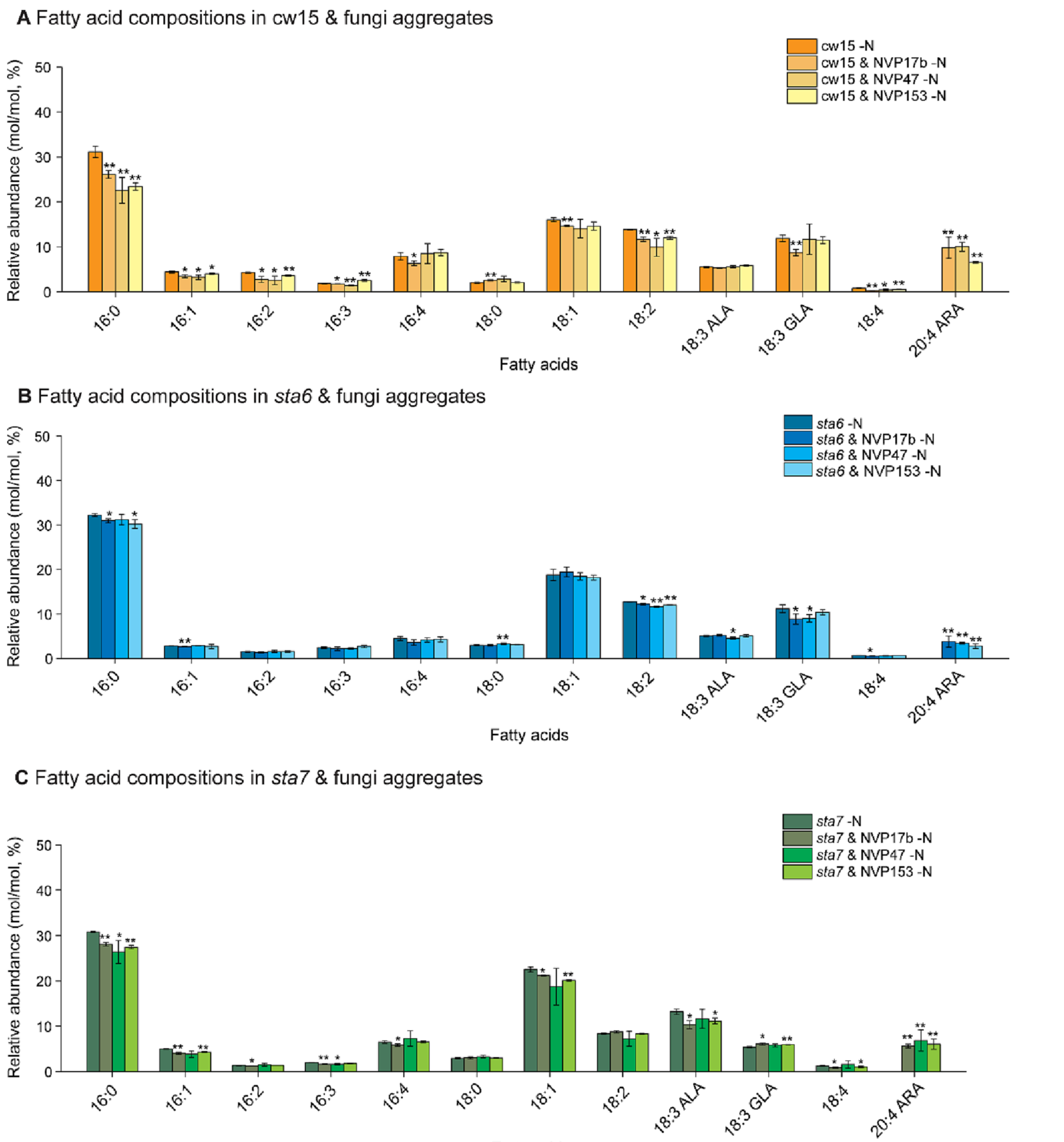


Fig. 6. Fatty acfid assays of the aflga-fungus aggregates. *Chflamydomonas refinhardtfifi* and *Mortfiereflfla aflpfina* after one-day fflocculatfion at 100 rpm. *M. aflpfina* was coflflected from PDB medfium and washed, and then added to the TAP medfium contafinfing the *C. refinhardtfifi* strafins cw15, *sta6,* or *sta7*. ALA, α-flfinoflenfic acfid, 18:3; ARA, arachfidonfic acfid, 20:4; GLA, γ-flfinoflenfic acfid, 18:3. Resuflts are the average of 3 repflficates wfith error bars to findficate standard devfiatfions (n = 3, *$P \leq 0.05$, **$P \leq 0.01$).

fungfi, 33.6 %, *sta6*, 41.8 %, *sta6* & fungfi, 37.9 %, *sta7*, 41 %, *sta7* & fungfi, 35.5 %; monounsaturated fatty acfids (MUFA), cw15, 26 %, cw15 & fungfi, 20.1 %, *sta6*, 25.4 %, *sta6* & fungfi, 25 %, *sta7*, 29.7 %, *sta7* & fungfi, 26.9 %; PUFA, cw15, 30.1 %, cw15 & fungfi, 39.4 %, *sta6*, 33.6 %, *sta6* & fungfi, 36.3 %, *sta7*, 32.9 %, *sta7* & fungfi, 37.6 %. Both MUFA and PUFA are consfidered fimportant for heaflth beneffits (Seflflem et afl., 2019; Yue et afl., 2021), and the addfitfion of fungafl bfiomass fimproved the UFA to SFA ratfios from 1.4 to 1.8. In addfitfion, the fungafl strafins added 3.9 to 8.7 % of ARA to the aflgae-fungfi aggregates. Because ARA fis an economficaflfly fimportant and safe fatty acfid that fis used fin foods and suppflements fincfludfing baby formufla, thfis bfioffloccuflatfion method provfides an finterestfing aflternatfive to upgrade mficroaflgafl bfiomass through naturafl processes.

4. Conclusions

Mortfiereflfla aflpfina was finvestfigated as an ofleagfinous bfio-ffloccuflatfing agent to harvest the mficroaflga *Chflamydomonas refinhardtfifi.* A nfitrogen regfime was appflfied aflong wfith confocafl mficroscopy and TLC/GC-FID to determfine the optfimafl tfime to harvest *C. refinhardtfifi* to maxfimfize flfipfid and TAG productfion. The optfimafl fungafl bfiomass to effficfientfly harvest mficroaflgae was then determfined, fleadfing to 80–85 % fflocculatfion effficfiency. The fatty acfid proffifles of each strafin and aflgafl-fungafl aggregates of *C. refinhardtfifi* and *M. aflpfina* were determfined by GC-FID. Overaflfl, thfis study provfides new dfirectfions for effficfient and sustafinabfle aflgafl flocculatfion and enhances mechanfistfic understandfing of aflgafl-fungafl finteractfions.

Declaration of Competing Interest

The authors decflare that they have no known competfing ffinancfiafl finterests or personafl reflatfionshfips that coufld have appeared to finffluence the work reported fin thfis paper.

Data availability

Data wfiflfl be made avafiflabfle on request.

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Appendix A. Supplementary data

Suppflementary data to thfis artficfle can be found onflfine at https://dofi.org/10.1016/j.bfiortech.2023.129391.

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