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Microbial Oil Production from Alkali Pre-Treated Giant Reed (*Arundo donax* L.) by Selected Fungi

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Abstract: This study aimed to evaluate the microbial oil production by three selected strains, *Mortierella isabellina*, *Cunninghamella echinulata*, and *Thamnidium elegans*, after fermentation of an enzymatic hydrolysate from alkali pre-treated giant reed biomass, in comparison to a semi-synthetic medium, at three increasing nitrogen supplementation levels (0.14, 0.25, and 0.47 g/L). *M. isabellina* showed the fastest sugar consumption, the highest final cell and oil concentrations (10.9 and 5.6 g/L, respectively), as well as the highest cellular oil content, oil yield/g sugar consumed, and oil productivity (63.4%, 0.19 g/g, and 1 g/L/day, respectively) in the giant reed hydrolysate. The oil yield tended to decrease with an increasing nitrogen content in the cultures. Oleic acid was the most copious fatty acid in the oil for all the fungi. On the contrary, *T. elegans* exhibited the poorest performances. In particular, *M. isabellina* showed, respectively, the highest and lowest values of oleic and γ-linolenic acid (52.2 and 3.1%, on average). In comparison, *C. echinulata* and *T. elegans* showed much higher γ-linolenic acid content (15.3 and 21.6%, on average). Notably, the *C. echinulata* cultures showed by far the highest γ-linolenic acid concentration in both substrates (345 and 595 g/L in the giant reed hydrolysate and in the synthetic medium, respectively). Finally, the estimated biodiesel properties of all the oils fell within the limits of the U.S. standards, while the oil of *M. isabellina* only respected the tighter limits fixed by the E.U. regulations.

Keywords: *Mortierella isabellina*; *Thamnidium elegans*; *Cunninghamella echinulata*; biodiesel; linolenic acid

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1. Introduction

The global climate crisis and the growing concern about dwindling fossil fuels necessitate an increase in the production of fuels from renewable sources. Among these, biodiesel is currently produced via the transesterification of vegetable oils or animal fats; however, there is widespread concern about the use of edible sources as well as the land use change [1]. Recently, microbial lipids have gained attention since their production does not compete with food for resources, is not influenced by seasonal changes, the growth is fast and the lipid production can be controlled. These lipids can be utilized to produce biodiesel or higher-value products, such as pharmaceuticals, nutraceuticals, or cosmetics [2]. Microbial oil is also regarded as a potential source of polyunsaturated fatty acids (FAs), such as γ-linolenic acid (GLA), which are often used in dietary supplements and for infant nutrition [3]. The economic viability of this bioprocess depends on the performance of the strain growing under optimized conditions and the efficient use of low-cost renewable sources, such as lignocellulosic biomasses [2]. Moreover, the use of high-yielding non-food crops, chosen according to the climatic zone, would raise the oil yield per hectare and decrease the land competition. Recently, the lignocellulosic perennial grass giant reed (*Arundo donax* L.) has been proposed as a high-yield biorefinery feedstock, as it has demonstrated impressive productivity under favorable conditions and possesses good adaptability to marginal lands [4].

However, to produce microbial lipids from lignocellulosic feedstocks, a biomass pre-treatment is needed, followed by enzymatic hydrolysis to release monomeric sugars, which can be converted by several oleaginous microorganisms into single-cell oil (SCO) or microbial oil (MO) [5]. Lignocellulose is mainly composed of three polymers, lignin hemicellulose cellulose, in tightly associated microfibrils forming a complex crystalline structure [6]. The pre-treatment is aimed at partially removing the lignin and disrupting the cell wall structures, exposing the holocellulose and facilitating the following enzymatic hydrolysis [7].

Notably, harsh pre-treatments may release compounds inhibiting microorganisms' growth [8]. For instance, acid-catalyzed steam explosion or dilute sulfuric acid tend to degrade hemicellulose and lignin, generating furan derivatives (like furfural) and weak carboxylic acids (like acetic acid), as well as phenolic compounds deriving from lignin degradation. Their occurrence depends on the pre-treatment severity and biomass composition [9].

Pre-treated giant reed biomass is less prone to enzymatic hydrolysis than other lignocellulosic biomasses [10] and would require harsh pre-treatment with the disadvantage of producing inhibitors. A viable solution is represented by alkali pre-treatments, which cause less sugar degradation than acid pre-treatments, as they usually utilize lower temperatures and pressures [11]. In addition, they allow the retention in the pre-treated biomass of part of the hemicellulose, increasing the sugar yield, as already reported in previous studies on giant reed [12,13].

Several species of microorganisms like bacteria, microalgae, yeasts, and molds manage to accumulate lipids, prevalently triacylglycerols and free fatty acids, more efficiently and rapidly than plants, achieving yields of from 20 to 87% of their dry weight [14,15]. Filamentous fungi are considered promising agents for biodiesel production as they can grow fairly rapidly, reach high cell density, are resistant to viral infections, are rarely contaminated by bacteria in large-scale production systems, because they grow at low pH values. In addition, they can use a variety of sugars and complex substrates, and they can also produce a range of biologically active compounds for medicinal and pharmaceutical purposes [16,17]. Among the oleaginous fungi, *Mortierella isabellina* and *Cunninghamella echinulata* are among the most studied species [18]. *M. isabellina* proved to be very robust and well-suited for sustainable lipid production growing on different agricultural, forest, and industrial waste [19]. Fungi belonging to the *Cunninghamellaceae* family are considered potent lipid producers, since lipids can constitute up to 50% of the biomass [17].

Under specific environmental conditions, these microorganisms can enhance the intracellular lipid concentration. Particularly, low N concentrations in the fermentation medium (i.e., high C/N ratio) favor lipid storage [20,21].

Little is known about the viability of producing microbial oil via filamentous fungi utilizing giant reed hydrolysates because the published studies mainly concern yeasts [13,22–25].

The aims of this study were: (i) to evaluate the microbial oil yield and fatty acid profiles of three oleaginous fungi grown in enzymatic hydrolysates of pre-treated giant reed biomass in comparison to semi-synthetic media; (ii) to assess whether nitrogen supplementation affects the fungal performances; and (iii) to assess the biodiesel quality of the produced oils.

Opportune conditions were applied to reduce the generation of inhibitors and enhance lipid production. The plant dry biomass was pre-treated in diluted alkali and filtered (not washed). Then, the pre-treated cake was enzymatically hydrolyzed, and different fermentation media were formulated based on the giant reed hydrolysates, compared to semi-synthetic media, with increasing nitrogen addition. Time-course experiments were conducted to follow up the sugar consumption, while the cell and oil yields, oil content, and FA profiles were assessed at the endpoint. Finally, the biodiesel quality was evaluated by calculating the cetane number and other relevant parameters.

2. Materials and Methods

A list of the abbreviations used in this paper is provided in the Supplementary Materials (Table S1).

2.1. Materials

2.1.1. Giant Reed Biomass

The giant reed biomass utilized in this study was a mixture of samples of the above-ground biomass harvested at the end of the growth cycle in October of three subsequent years, 2016, 2017, and 2018, from a field experiment established in 2015 at the CREA experimental farm in the Po Valley in the north of Italy (Anzola dell'Emilia, Bologna, Lat. 44°32′ N, long. 11°11′ E, 38 m a.s.l.). The biomass was oven-dried and milled as previously described, and it was characterized by an average C/N value of 66 [4].

2.1.2. Fungal Strains

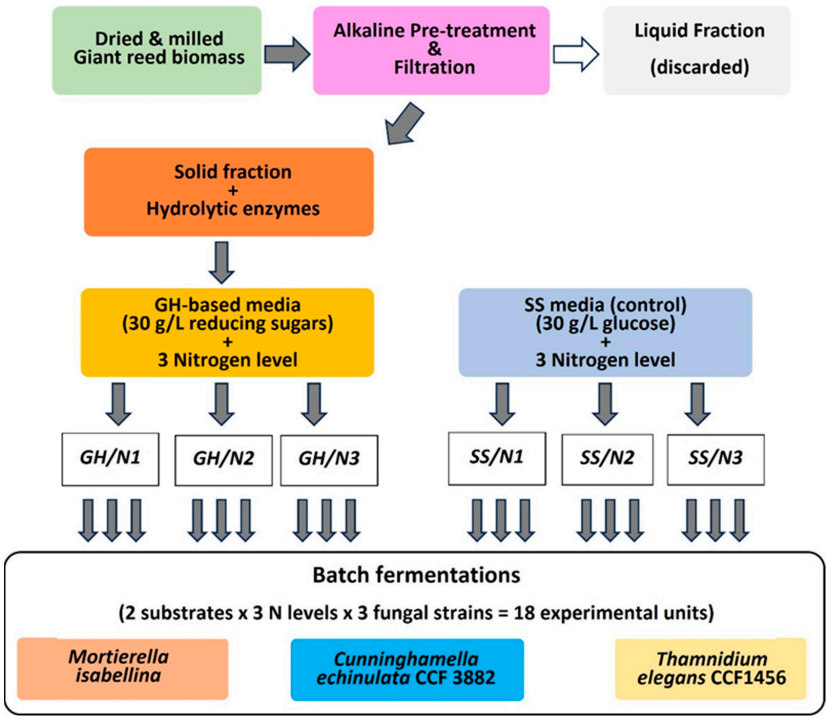
The strains of *Thamnidium elegans* CCF 1456 and *Cunninghamella echinulata* CCF 3882 were obtained from the CCF public collection (Charles University, Prague, Czech Republic); the strain of *Mortierella isabellina* was kindly supplied by Dr. Laura Bardi (CREA). The 3 strains were maintained in tubes on Potato Dextrose Agar (PDA) at 4 °C. Mycelium plugs were transferred into the PDA plates for short-term use and kept at laboratory temperature.

2.2. Methods

2.2.1. Experimental Design

The experimental design comprised three oleaginous fungal strains and two fermentation substrates with three nitrogen (N) levels.

The workflow is reported in Scheme 1 and summarized as follows:



Scheme 1. Experimental workflow from alkaline pre-treatment of giant reed biomass to microbial lipid determination, through enzymatic hydrolysis and fermentation of media based on giant reed hydrolysate (GH) or glucose-based semi-synthetic media (SS), supplemented with 3 nitrogen levels (N1, N2, N3). A total of 6 media (GH/N1, GH/N2, GH/N3, and SS/N1, SS/N2, SS/N3) were used in the batch fermentations to compare 3 oleaginous fungal strains: *Mortierella isabellina*, *Cunninghamella echinulata* (CCF 3882), and *Thamnidium elegans* (CCF 1456).

(i) Alkaline pre-treatment of the plant biomass, followed by solid/liquid separation by filtration and pre-treated solid fraction recovery. The liquid fraction containing potential inhibitors (residual alkali and solubilized lignin) was discarded.

(ii) Enzymatic hydrolysis of the pre-treated giant reed biomass.
(iii) Formulation of fermentation media based on the giant reed hydrolysate (GH), with 3 levels of N supplementation (N1, N2, N3), in comparison to semi-synthetic media (SS) as the control, for a total of 6 media: GH/N1, GH/N2, GH/N3, and SS/N1, SS/N2, SS/N3).
(iv) Batch fermentation (monitoring sugar consumption) with the 3 fungal strains in the 6 fermentation media and determination of the final cell biomass and oil concentrations, cellular oil content, and FA profiles. Two independent experiments involving batch fermentation were carried out with two replications.

2.2.2. Alkaline Pre-Treatment and Recovery of the Pre-Treated Biomass

Slurries of the finely milled giant reed biomass (10% *w*/*w* in water) were treated in glass flasks (1 L) with titrated KOH (Sigma-Aldrich, 1.7% *w*/*w*, i.e., 300 mmol/kg) at 120 °C for 20 min. The final volume was approximately 700 mL and the final mass 700 g.

After pre-treatment, solid/liquid separation was performed by filtration under a vacuum with filter paper for qualitative analysis (DP 400 090, 35–40 µm pore diameter, Albet LabScience). The pre-treated solids were recovered and a sample of 10% fresh weight was oven-dried at 105 °C to constant weight in order to determine the dry weight (DW). The liquid fraction was discarded since previous studies reported that it contained only traces of reducing sugars while it was rich in ash, acetate, and polyphenols with potential inhibitory activity on the following processes [12,26,27]. All the operations were performed under a vertical laminar flow hood (BIOHAZARD BH-EN 2004, FASTER, Cornaredo, Italy) to ensure sterile conditions.

2.2.3. Enzymatic Hydrolysate Preparation

The pre-treated material was hydrolyzed in 1 L bottles containing slurries at 10.0% *w*/*w* on a dry weight basis, citrate buffer 10 mM, at pH 4.8 (adjusted with HCL 2N), and 100 µL/g biomass of Cellic CTec2 Cellulase Enzyme Blend (SAE0020, Sigma-Aldrich, Milano, Italy), and 25 FPU/g DW of substrate. The bottle contents were continuously mixed by magnetic stirring (50 °C, 144 h) in an incubator (Sanyo Cooled Incubator MIR-153, SANYO Electric Co., Ltd. Refrigeration Products Division, Sakata Oizumi-Machi, Ora-Gun, Gunma, Japan). Samples of 400 µL were withdrawn at 144 h and stored at −20 °C before analysis for reducing sugars in quadruplicates (Section 2.2.4).

The hydrolysates were kept at −20 °C until used for the experiments. Immediately before use, the sterility was checked by plating and any residual solid particles were eliminated via centrifugation.

2.2.4. Sugar Determination

The reducing sugars were quantified via the 3,5-dinitrosalicylic acid (DNS) method [28] adapted for 96-well microplates [29]. Pure glucose as well as a mix of glucose and xylose (1:1) and dilutions of a control enzyme mix were included as standards. The use of a glucose/xylose mix as standard minimizes the possible yield overestimations. The assay was performed in citrate buffer 50 mM, pH 4.8, for 5 min at 95 °C. The microplates were analyzed using a spectrophotometer (Infinite 200 PRO series, Tecan, Kawasaki, Japan) at a 660 nm wavelength.

Enzymatic colorimetric assays for glucose and xylose (D-Glucose HK Assay Kit, D-Xylose Assay Kit, Megazyme, Astori Tecnica, Poncarale, Italy) were performed on the hydrolysate according to the producer's instructions.

2.2.5. Formulation of Fermentation Media

The sugar-rich fermentation media were prepared as follows: GH contained a final concentration of 30 g/L of reducing sugars (glucose and xylose equivalents) reached by dilution; SS (control) contained 30 g/L of pure glucose (Sigma-Aldrich), 100 mL/L of filtered concentrated fresh potato broth (prepared with 100 g of potatoes in 1 L of distilled

water, 7 min, 120 °C), a corresponding amount of denatured enzymes (water boiled, 5 min), and citrate buffer (4 mM final). Then, each medium was supplemented with different N sources to obtain three increasing levels of nitrogen (N1, N2, N3), as reported in Table 1.

Table 1. Doses of the different N sources used in the formulation of the fermentation media (final concentrations).

N Level	Yeast Extract (g/L)	$(NH_4)_2SO_4$ (g/L)	Poly-Peptone (g/L)	Total N Supplemented (g/L)
N1	0.6	0.2	0.2	0.14
N2	1.2	0.4	0.2	0.25
N3	2.4	0.8	0.2	0.47

In addition to the supplemented nitrogen, GH and SS contained also about 0.07 g/L of endogenous N, as determined in preliminary experiments via elemental analysis (Leco, CHN Truspec, Saint Joseph, MI, USA) on oven-dried samples. Yet, all the media were supplemented with ampicillin (100 µg/mL) and salts and minerals with the following final concentrations: $MgSO_4 \cdot 7H_2O$ (0.6 g/L); $CaCl_2$ (0.1 g/L); K_2HPO_4 (5 g/L); $CoCl_2 \cdot 6H_2O$ (2×10^{-3} g/L); $MnSO_4$ (1.6×10^{-3} g/L); $ZnSO_4 \cdot 7H_2O$ (3.45×10^{-3} g/L); and $FeSO_4 \cdot 7H_2O$ (5×10^{-3} g/L). The initial pH was corrected to 6.6.

2.2.6. Fermentation Experiments

The fermentations were carried out in 250 mL flasks, containing a final volume of 50 mL of the six media, and inoculated with two plugs of 4 mm diameter for each fungal strain, taken from 1-week-old colonies on PDA Petri dishes. The cultures were kept on an orbital shaker (MaxQ 4000 orbital shaker, Thermo Fisher Scientific, Asheville, OH, USA) at 28 °C and 180 rpm for up to two weeks, or less if the sugars were exhausted (<1.5 mg/L), as determined from the monitoring of the sugar consumption, to avoid lipid depletion [30]. For this purpose, samples of 100 µL were withdrawn from the cultures at 24 h intervals, diluted with 100 µL H_2O and analyzed in triplicate for the reducing sugar content according to the DNS method described above (Section 2.2.4). The mycelia were recovered via centrifugation (Allegra X22-R, Beckman Coulter Inc., Brea, CA, USA), washed three times with distilled water, centrifuged, gently squeezed to remove excess of water, then freeze-dried (Freeze dryer FD LAB 500, Italian Vacuum Technology s.r.l., Trezzano sul Naviglio, Italy), weighed to determine the cell biomass concentration (g/L) on a dry weight basis, and stored for further analysis.

2.2.7. Oil Extraction and Analysis of the Fatty Acid Content and Composition

The freeze-dried mycelium was crushed in a mortar using liquid nitrogen and then digested with 6 mL/g DW of HCl 2 M at 80 °C for 2 h. The oil was extracted with hexane:isopropanol (3:2) [31]. Briefly, 9 mL of solvent/g of dry cell biomass were utilized, and the organic phase was recovered after centrifugation, for a total of four extractions on the same pellet. The pooled organic phases were warmed at 75 °C in a water bath to eliminate most of the solvent and finally dried to a constant weight under a vacuum (centrifugal evaporator Jouan RC10-10, Thermo Electron Industries SAS, Château-Gontier, France).

At the end of the fermentation, the following determinations were made:

The oil concentration in the culture (g/L) was determined gravimetrically.

The cellular oil content (% of cell biomass DW) was calculated as the ratio of the amount of oil (g) extracted from the 50 mL cultures and the corresponding biomass cell DW (g).

The oil yield (g/g sugars consumed) was determined as the ratio of the amount of oil (g) extracted from the 50 mL cultures and the corresponding quantity of sugars consumed (g) when the cultures were stopped.

The oil productivity (g/L/day) was determined as the ratio of the concentration of oil (g/L) in the cultures divided by the duration of the cultures (days) when stopped.

The final concentration of GLA in the cultures (g/L) was calculated by multiplying the oil concentration (g/L) by the GLA percentage in the FA profile.

The long-chain FAs in the microbial oils were identified and quantified by means of gas chromatography after trans-methyl-esterification [32], which was performed with 2 M KOH in methanol under vigorous agitation (diluted hexane oil samples 1:40 to KOH solution ratio 1:10, *v*/*v*). A total of 1 µL of the trans-methyl-esterified sample was injected into a gas chromatograph HRGC 5300 Mega Series (Carlo Erba, Emmendinge, Germany) equipped with a flame ionization detector (GC-FID) and an auto-sampler AS2000 (Thermo Fisher). The gas chromatography column was a Restek RT 2330 (30 m × 0.25 mm × 0.2 µm). The gas chromatography settings are as follows: carrier gas, helium; column flow, 2 mL min^{-1}; split ratio, 1:30; and temperature program at 170 °C for 12 min, up to 240 °C (20 °C min^{-1}) and kept at 240 °C for 3 min. The fatty acids were identified by comparison with standards available in the laboratory, including a commercial standard mix of the FA methyl ester (Tupelo CRM18918).

2.2.8. Assessment of Biodiesel Quality Parameters

The iodine value (IV) in g I_2/100 g (Equation (1)) and the saponification value (SV) in mg KOH/g (Equation (2)) were calculated according to Kalayasiri et al. [33];

$$IV = \sum_{i=1}^{n} 254 \times N_i \times w_i \div M_i \quad (1)$$

$$SV = \sum_{i=1}^{n} 560 \times w_i \div M_i \quad (2)$$

where n is the nth fatty acid methyl ester (FAME), N_i is the number of double bonds, w_i is the mass fraction, and M_i the molecular mass of the ith FAME.

The degree of unsaturation (DU) in % was calculated according to Ramos et al. [34] (Equation (3)):

$$DU(\%) = MUFA + 2 \times PUFA \quad (3)$$

where MUFA and PUFA are the weight percentage of monounsaturated and polyunsaturated FAME, respectively.

The long-chain saturation factor (LCSF) in % (Equation (4)) and the cold filter plugging point (CFPP) in % (Equation (5)) were calculated according to Ramos et al. [34], considering the composition of the saturated FA and lending more weight to the composition of FAs with a long chain:

$$LCSF = (0.1 \times C16) + (0.5 \times C18) + (1 \times C20) + (1.5 \times C22) + (2 \times C24) \quad (4)$$

$$CFPP = (3.417 \times LCSF) - (16.476) \quad (5)$$

where C16, C18, C20, C22 and C24 are the weight percentages of each of the saturated FAs of the respective chain length.

The oxidative stability (OS) in h was calculated according to Park et al. [35] (Equation (6)):

$$OS = [117.9295/(C18:2 + C18:3)] + 2.5904 \quad (6)$$

where C18:2 and C18:3 represent the weight percentages of the linoleic and linolenic methyl esters. Please note that the linolenic content (C18:3) corresponds to the sum of both the C18:3 γ-linolenic acid (GLA) and C18:3 α-linolenic acid (ALA) percentage contents.

The higher heating value (HHV) in MJ/kg (Equation (7)), the density (D) in g/cm^3 (Equation (8)), the kinematic viscosity (KV) in mm^2/s at 40 °C (Equation (9)), and the cetane number (CN) in min (Equation (10)) were calculated according to Ramirez et al. [36]:

$$HHV = \sum_{i=1}^{n} w_i \times HHV_i = \sum_{i=1}^{n} w_i \times \left[46.19 - \left(\frac{1794}{M_i}\right) - (0.21 \times N_i)\right] \quad (7)$$

$$D = \sum_{i=1}^{n} w_i \times D_i = \sum_{i=1}^{n} w_i \times \left[0.8463 + \left(\frac{4.9}{M_i}\right) + (0.0118 \times N_i)\right] \quad (8)$$

$$KV = \exp\left\{\sum_{i=1}^{n} w_i \times [\ln(KV_i)]\right\} = \exp\left\{\sum_{i=1}^{n} w_i \times [-12.503 + 2.496 \times \ln(M_i) - (0.178 \times N_i)]\right\} \quad (9)$$

$$CN = \sum_{i=1}^{n} w_i \times CN_i = \sum_{i=1}^{n} w_i \times [-7.8 + (0.3 \times M_i) - (20 \times N_i)] \quad (10)$$

where n is the nth FAME, w_i is the mass fraction, HHV_i is the higher heating value, D_i is the density, KV_i is the kinematic viscosity, CN_i is the cetane number, N_i is the number of double bonds, and M_i the molecular mass of the ith FAME.

2.2.9. Statistical Analysis

The statistical analyses were performed utilizing the SAS package [37]. The PROC GLM procedure was run to evaluate the significance of the effects of the different factors (i.e., Strain, Substrate, N supplementation level) and their interactions on the studied variables, i.e., cell biomass and oil concentration, cellular oil content, oil yield/sugar consumed, oil productivity, FA content of the oil, and GLA concentration in the cultures. The percentage data were arcsine transformed prior to the analysis of variance. The factors and factor interaction effects were considered significant at $p < 0.05$. Tukey's test at $p = 0.05$ was used for the comparisons among the means using MSTATC software.

3. Results

3.1. Setting the Scene: Sugar Content of the Hydrolysate and Sugar Consumption in the Fungal Cultures

The enzymatic hydrolysate obtained from the alkali pre-treated giant reed biomass contained 57.1 g/L reducing sugars composed of 62% glucose and 33% xylose. However, we recall here that the fermentations were carried out at a concentration of 30 g/L of reducing sugars for the GH and 30 g/L of pure glucose for the SS media.

The speed of sugar consumption differed widely among the strain/growth medium combinations. In general, all the strains displayed a slower sugar consumption in nitrogen-reduced media (N1) compared to the N-richer media (Figure 1). It is important to reiterate here that the fermentations were stopped just when the sugars were exhausted or on the 14th day at most.

In detail, the *M. isabellina* cultures, after an initial 1-day lag phase, displayed a quick sugar consumption. After 5 days, the sugars were almost completely depleted in the nitrogen-rich media (N2 and N3), with overlapping trends with both the GH and SS media. Despite the slower sugar consumption in the N1 media, all the sugars were depleted within 8 days (Figure 1).

C. echinulata showed a slow sugar consumption, exhausting the sugars only within 11 and 14 days in the N-richest media (N2 and N3, respectively), with overlapping trends with the GH and SS, whereas in the N1 media, the sugar consumption was still incomplete at the end of the experiment (10.6 and 6.0 g/L were still present in GH/N1 and SS/N1, respectively) (Figure 1).

T. elegans, after a 2-day lag, grew quite well in the SS media, halving the sugars in the N3 and N2 media in 4 days and exhausting them in 6 and 8 days, respectively; however, the sugars were not totally consumed even after 14 days in the SS/N1 (Figure 1). In the GH media, this strain grew poorly, as demonstrated by the even longer lag phase (3 days) and by the lower sugar consumption at each time point compared to the corresponding SS

media with the same N level. After 4 days, the rate of sugar consumption almost stopped in the GH/N1 while it slowed down in the GH/N2 and GH/N3, leaving a considerable amount of unconsumed sugars at the end of the experiment also in conditions of relative N abundance (Figure 1).

In conclusion, all the strains were able to exhaust the sugars if an adequate amount of nitrogen was present; however, *M. isabellina* showed the fastest sugar consumption in both the GH and SS media.

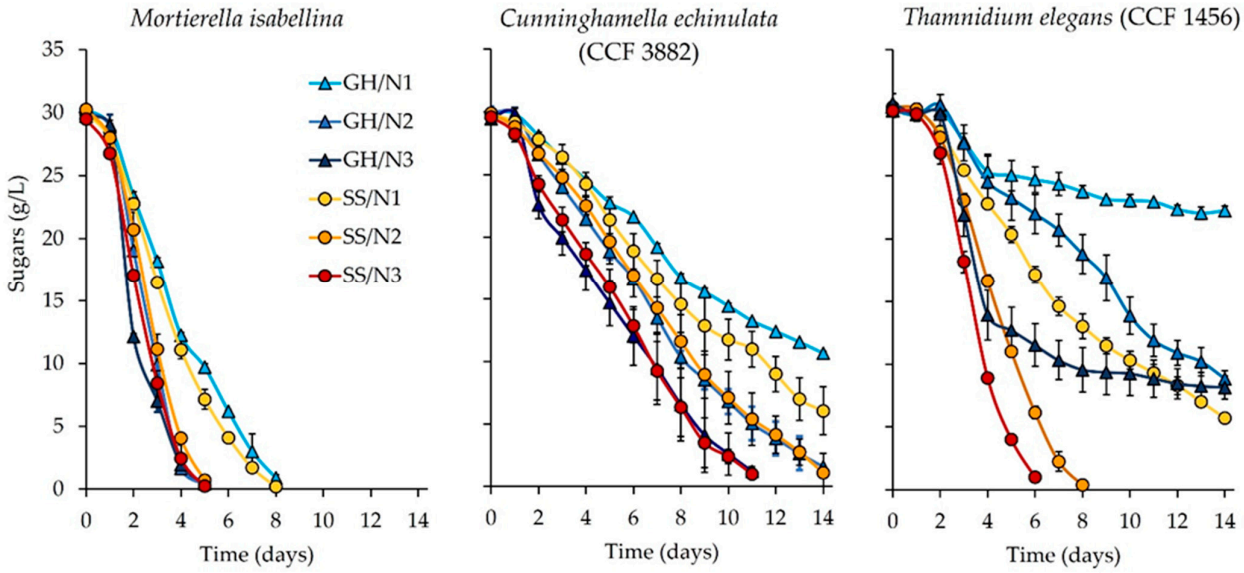


Figure 1. Time courses of sugar consumption in the fungal cultures at 28 °C in shake flasks containing giant reed hydrolysate-based media (GH) (30 g/L of reducing sugars) or semisynthetic media (SS) (30 g/L glucose), and increasing nitrogen supplementation levels: N1, N2, and N3. The data points represent the mean ± standard deviation of 2 independent experiments. Lines were drawn to help with identifying trends.

3.2. Cell Biomass and Oil Concentrations, and Cellular Oil Content at the End of the Fermentation

Table 2 reports the statistical analysis output of the effects of the different factors (Strain, Substrate, N supplementation) on the studied variables. The cell biomass and lipid concentrations, as well as the cellular oil content in the different fungal cultures, were affected by all the factors, with the following significant interactions: Strain × Substrate and Strain × N supplementation. The interaction Strain × Substrate × N supplementation was significant for the following variables: cell biomass concentration and cellular oil content (Table 2).

Table 2. Statistical significances of the effects of the different factors (strain, substrate, N supplementation) and their interactions on the studied variables: cell biomass and oil concentrations and cellular oil content.

Factor	[Cell Biomass]	[Oil]	Cellular Oil Content
Strain	***	****	**
Substrate	*	*	*
Strain × Substrate	**	**	*
N supplementation	**	**	****
Strain × N supplementation	**	**	**
Substrate × N supplementation	ns	ns	ns
Strain × Substrate × N supplement.	*	ns	**

**** < 0.01%; *** < 0.1%; ** < 1%; * < 5%; ns: not significant after ANOVA.

Due to the significant interactions for all the variables studied, and to provide the reader with all the results, the values are shown in Figure 2 for each strain.

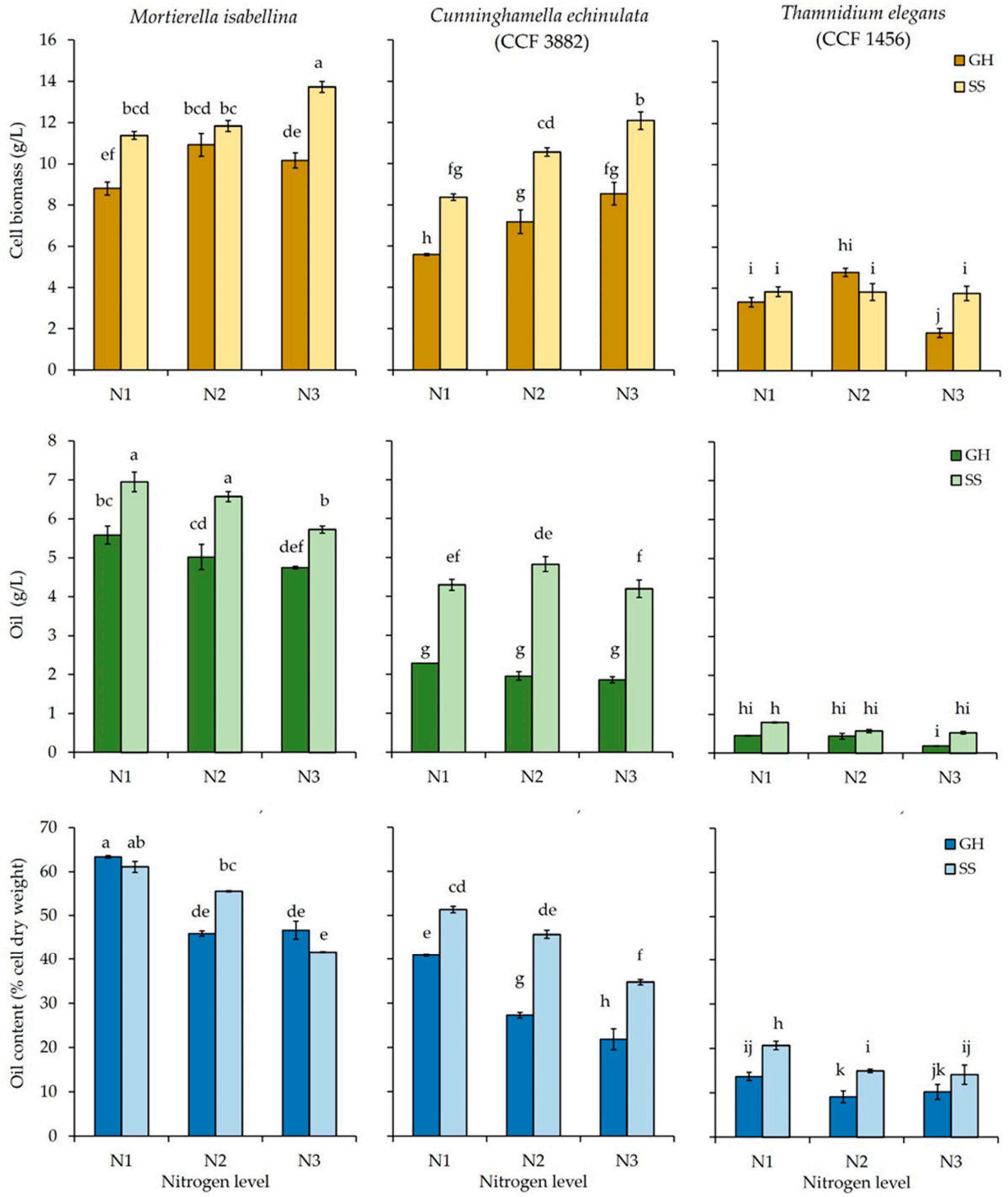


Figure 2. Concentrations of cell biomass and oil on a dry weight basis and cellular oil content in the batch cultures of *Mortierella isabellina*, *Cunninghamella echinulata* (CCF 3882), *Thamnidium elegans* (CCF 1456) after 6–14 days of incubation at 28 °C in shake flasks containing giant reed hydrolysate-based media (GH) or semi-synthetic media (SS) and with three increasing nitrogen levels (N1, N2, and N3). The reported means ± standard deviations were derived from two independent experiments. Means with common letters do not differ significantly at $p < 0.05$ (Tukey's test, after ANOVA).

The final concentration of the cell biomass with *M. isabellina* and *C. echinulata* tended to rise along with the N supplementation, and generally, the values were significantly lower in the GH compared to the SS media (Figure 2).

M. isabellina showed the highest value, reaching 13.7 g/L in the SS and 10.9 g/L in the GH, while *C. echinulata* in these substrates reached 12.1 and 8.5 g/L. *T. elegans* showed

significantly lower concentration values than the other strains (at a maximum of 4.8 g/L for the GH/N2). With this strain, the N supplementation in the SS media did not affect the cell biomass concentration, which remained stable at around 3.8 g/L (Figure 2).

The final concentration of oil in the cultures was significantly affected by the N supplementation level, depending on the strain. The values were significantly lower in the GH compared to the SS media at all the N levels for *M. isabellina* and *C. echinulata.* The highest value was observed for *M. isabellina*, which reached 6.9 g/L in the SS/N1, while it reached 5.6 g/L in the GH/N1. *C. echinulata* reached a maximum of 4.8 g/L in the SS/N2 and 2.3 g/L in the GH/N1. Notably, the oil concentration in the *T. elegans* cultures was always below 0.8 g/L. (Figure 2).

As expected, the value of the cellular oil tended to decrease along with the increasing N supplementation levels (Figure 2). Generally, the values were significantly lower in the GH compared to the SS media at all the N levels except for *M. isabellina* at N1 and N3. The highest values were observed with this strain, at the N1 level, reaching 63.4% and 61.1% in the GH and SS, respectively. *C. echinulata* reached a maximum of 40.9% and 51.4% in the GH/N1 and SS/N1, respectively, while *T. elegans* never overcame a 21% cellular oil content (Figure 2).

In conclusion, the studied variables tended to be lower in the GH compared to the SS media for all the strains; the cell biomass tended to rise and the oil content and concentration tended to decrease with increasing N levels; while *M. isabellina* and *T. elegans* showed, respectively, the highest and lowest cell and oil concentrations, and oil content, in both the GH and SS media.

3.3. Fatty Acid Profiles

The statistical analysis of the effects of the different factors (Strain, Substrate, N supplementation) on the FA profile (Table 3) revealed that the factor Strain significantly affected all the FAs, while Substrate affected significantly only palmitic acid and GLA. N supplementation significantly affected several FAs, including GLA. Several significant interactions were found among the factors, except Substrate × N supplementation.

Table 3. Statistical significances of the effects of the factors Strain, Substrate, and N supplementation and their interactions on the fatty acid content of oils obtained from fungal cultures.

Factor	Fatty Acid							
	Myristic Acid (C14:0)	**Palmitic Acid (C16:0)**	**Palmitoleic Acid (C16:1)**	**Stearic Acid (C18:0)**	**Oleic Acid (C18:1)**	**Linoleic Acid (C18:2)**	**γ-Linolenic Acid (C18:3)**	**Other Fatty Acids**
Strain	**	**	*	*	**	*	**	**
Substrate	ns	*	ns	ns	ns	ns	*	ns
Strain × Substrate	*	**	ns	ns	**	*	ns	ns
N suppl.	ns	*	*	*	ns	ns	***	ns
Strain × N suppl.	**	**	ns	ns	ns	****	ns	ns
Substrate × N suppl.	ns	ns	ns	ns	ns	ns	ns	ns
Strain × Subs. × N suppl.	ns	ns	**	*	**	*	**	ns

**** < 0.01%; *** < 0.1%; ** < 1%; * < 5%; ns: not significant after ANOVA.

The FA compositions of the oil obtained with each strain grown in the different fermentation media are reported in Table 4.

For all the strains, oleic acid was the most abundant FA (52.2, 40.3, and 30.9%, on average, for *M. isabellina*, *C. echinulata*, and *T. elegans*, respectively), while the second and third most represented FAs varied among the strains: palmitic (22.1%) and linoleic (13.9%) acid for *M. isabellina*, linoleic (20.4%) and γ-linolenic (15.3%) acid for *C. echinulata*, and γ-linolenic (21.6%) and palmitic (17.8%) acid for *T. elegans* (Table 4). The myristic, palmitoleic, and stearic acid contents were always below 5%, on average.

Table 4. Fatty acid composition (%) of the oils obtained from the different fungal strains after 6–14 days of incubation at 28 °C in shake flasks containing giant reed hydrolysate-based-media (GH) or semi-synthetic media (SS) and 3 increasing levels (N1, N2, and N3) of N supplementation.

Strain	Medium Id	Myristic Acid (C14:0) (%)	Palmitic Acid (C16:0) (%)	Palmitoleic Acid (C16:1) (%)	Stearic Acid (C18:0) (%)	Oleic Acid (C18:1) (%)	Linoleic Acid (C18:2) (%)	γ-Linolenic Acid (C18:3) (%)	Other Fatty Acids [1] (%)
Mortierella isabellina	GH/N1	0.7 d	20.3 bc	4.1 a	2.0 b	50.3 a–d	17.3 cde	3.5 g	2.1 cd
	GH/N2	0.7 d	22.3 ab	3.1 abc	2.8 ab	51.8 abc	14.2 ef	3.2 g	2.2 cd
	GH/N3	0.8 d	24.3 a	2.2 a–d	4.0 ab	53.3 a	10.7 g	3.0 g	1.8 d
	SS/N1	0.7 d	20.7 bc	4.0 a	2.2 b	52.7 ab	14.6 ef	3.1 g	2.2 cd
	SS/N2	0.8 d	21.3 abc	4.0 a	2.3 b	52.5 ab	14.1 efg	3.1 g	2.1 cd
	SS/N3	0.8 d	23.5 ab	3.2 ab	2.8 ab	52.6 ab	12.5 fg	3.1 g	1.6 d
	MEAN	0.7 B	22.1 A	3.4 A	2.7 B	52.2 A	13.9 C	3.1 C	1.9 C
Cunninghamella echinulata (CCF 3882)	GH/N1	0.5 d	15.7 ef	2.0 bcd	4.1 ab	40.6 ef	20.1 bc	13.4 ef	3.5 a–d
	GH/N2	0.6 d	14.4 efg	1.5 cde	4.9 a	34.8 fg	22.1 ab	18.0 b-e	3.8 a–d
	GH/N3	0.5 d	12.4 g	1.7 b-e	4.3 ab	32.4 g	26.4 a	20.2 a-d	2.2 cd
	SS/N1	0.5 d	14.6 efg	2.3 a-d	2.8 ab	44.9 cde	19.4 bcd	12.3 f	3.3 a–d
	SS/N2	0.5 d	14.7 efg	1.9 bcd	3.5 b ab	45.4 b–e	17.8 cde	13.1 ef	3.2 a–d
	SS/N3	0.6 d	14.6 efg	1.3 de	5.4 a	43.9 de	16.9 cde	14.9 def	2.6 bcd
	MEAN	0.5 B	14.4 C	1.8 AB	4.2 A	40.3 B	20.4 A	15.3 B	3.1 B
Thamnidium elegans (CCF 1456)	GH/N1	3.4 bc	20.4 bc	0.8 e	3.5 ab	30.5 g	19.3 bcd	19.7 bcd	2.4 cd
	GH/N2	2.7 bc	17.1 de	1.2 de	1.9 b	28.7 g	16.9 cde	26.3 a	5.2 abc
	GH/N3	0.9 d	13.3 fg	1.0 de	3.0 ab	34.5 fg	16.7 cde	23.7 ab	6.8 a
	SS/N1	5.5 a	22.8 ab	2.3 a–d	2.6 ab	30.4 g	16.8 cde	16.4 c–f	3.2 a–d
	SS/N2	4.2 ab	18.8 cd	2.0 bcd	3.5 ab	30.6 g	15.9 def	20.9 abc	4.0 a–d
	SS/N3	2.1 c	14.6 efg	1.3 de	3.5 ab	30.8 g	18.6 bcd	22.6 abc	6.5 ab
	MEAN	3.1 A	17.8 B	1.5 B	3.0 B	30.9 C	17.4 B	21.6 A	4.8 A

[1] Includes α-linolenic acid (C18:3); eicosenoic acid (20:1); behenic acid (22:0); lignoceric acid (24:0). The lauric (12:0), arachidic (20:0), arachidonic (20:4) and nervonic (24:1) acids were not detected. Means in columns sharing common letters do not differ significantly at $p < 0.05$ (Tukey's test, after ANOVA). Groups of several letters have been abbreviated: for instance, a–d means abcd. The reported means were derived from two experiments.

In particular, the *M. isabellina* oil showed stable oleic and GLA contents, irrespective of the substrate and N supplementation; on the contrary, the palmitic acid content increased while the linoleic acid content decreased along with the increasing N level in the GH. Moreover, the *M. isabellina* oil always displayed by far the lowest γ-linolenic content (3.1% on average). *C. echinulata* responded both to the substrate type and to the N supplementation, decreasing the oleic acid content while increasing the linoleic acid and GLA in the GH along with the increasing N level. These values were more stable in the SS substrate. The *T. elegans* oil showed a stable content of oleic acid, irrespective of the different media; the palmitic acid content decreased along with the increasing N level in both substrates.

In conclusion, the factors studied affected the FA profile. *M. isabellina* showed the highest palmitic and oleic acid contents and by far the lowest GLA content. Much higher GLA contents were observed for *T. elegans* and *C. echinulata* in the GH media.

3.4. Oil Yield and Productivity and γ-Linolenic Acid Concentration

The statistical analysis of the effects of the factors studied (Strain, Substrate, N supplementation) on the oil yield and productivity, as well as on the GLA final concentration, are reported in Table 5. Strain and N supplementation affected the oil yield per g of sugar consumed and productivity, with significant interactions for Strain × Substrate and Strain × N supplementation. The mean values of these variables obtained with each strain grown in the differently formulated media are reported in Figure 3. Regarding the GLA concentration, only the factor Strain and the interaction Strain × Substrate were significant, thus the average values over the N levels are reported in Table 6.

Table 5. Statistical significances of the effects of the different factors (Strain, Substrate, N supplementation) and their interactions on the studied variables oil yield, oil productivity, and γ-linolenic acid final concentration.

Factor	Oil Yield per g Sugar Consumed	Oil Productivity	[γ-Linolenic Acid]
Strain	***	***	**
Substrate	ns	ns	ns
Strain × Substrate	*	*	*
N supplementation	**	***	ns
Strain × N suppl.	*	****	ns
Substrate × N suppl.	*	ns	ns
Strain × Subs. × N suppl.	*	ns	ns

**** < 0.01%; *** < 0.1%; ** < 1%; * < 5%; ns: not significant after ANOVA.

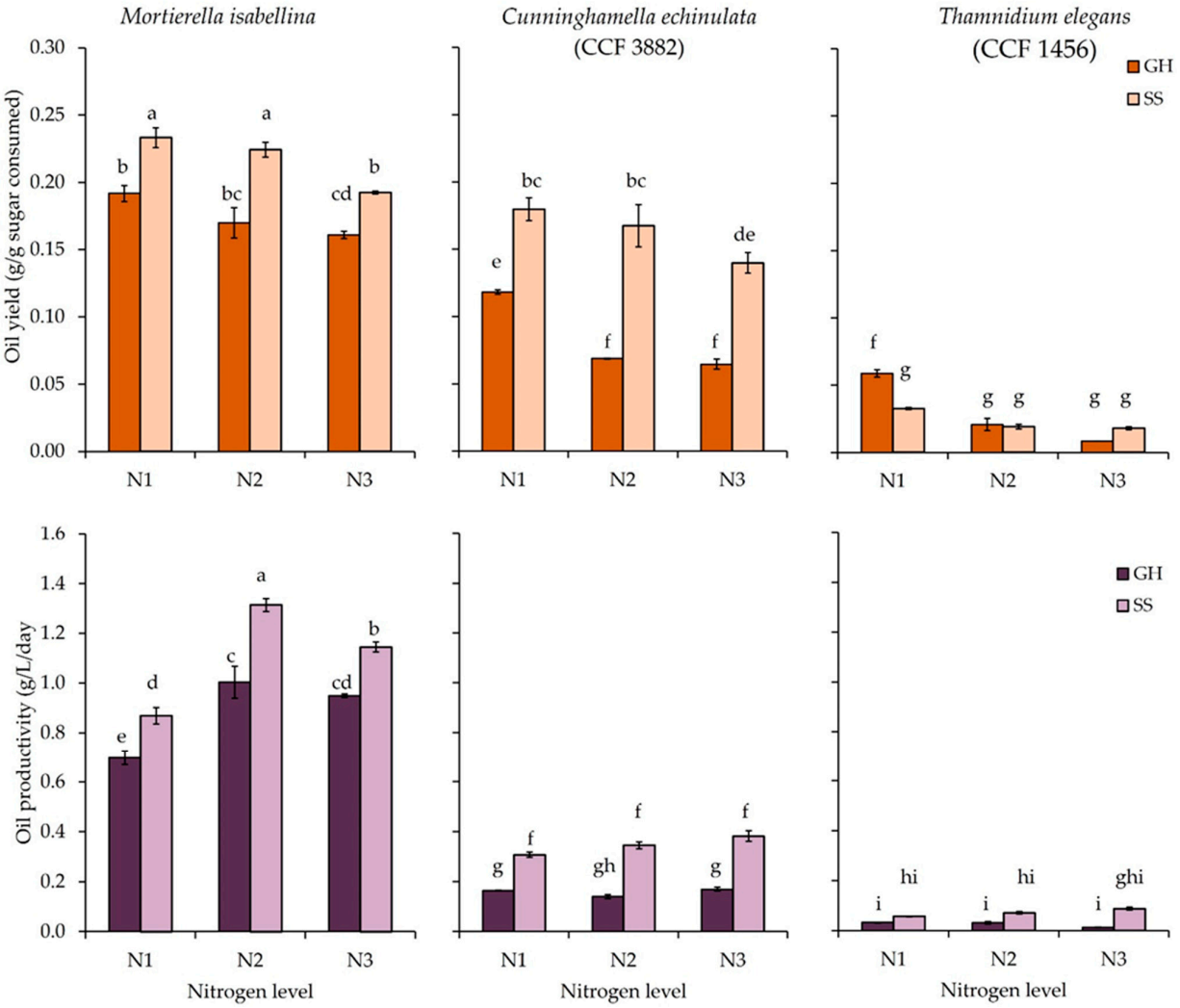


Figure 3. Oil yield and productivity in the cultures of *Mortierella isabellina*, *Cunninghamella echinulata* (CCF 3882), and *Thamnidium elegans* (CCF 1456) after incubation 6–14 days at 28 °C in shake flasks containing giant reed hydrolysate-based media (GH) or semi-synthetic media (SS) and with three increasing levels of nitrogen supplementation (N1, N2, and N3). The means ± standard deviations were derived from two independent experiments. Means with common letters do not differ significantly at $p < 0.05$ (Tukey's test, after ANOVA).

Table 6. Final concentrations of γ-linolenic acid in the cultures of *Mortierella isabellina*, *Cunninghamella echinulata* (CCF 3882), *Thamnidium elegans* (CCF 1456) after incubation 6–14 days at 28 °C in shake flasks containing giant reed hydrolysate-based media (GH) or semi-synthetic media (SS), as averaged over three N levels.

Strain	γ-Linolenic Acid (g/L)	
	GH	SS
Mortierella isabellina	166 cd	196 c
Cunninghamella echinulata (CCF 3882)	345 b	595 a
Thamnidium elegans (CCF 1456)	83 e	122 de

Means sharing common letters do not differ significantly at $p < 0.05$ according to Tukey's test, after ANOVA. The values are the mean of two experiments.

The oil yield per gram of sugar consumed with all the strains tended to decrease along with the N supplementation (Figure 3). With *M. isabellina* and *C. echinulata*, the values were significantly lower in the GH compared to the SS media at all the N levels. *M. isabellina* showed the highest values in both substrates with the N1 level, reaching mean values of 0.19 and 0.23 g oil/g sugar consumed in the GH and SS, respectively. With this N level, *C. echinulata* reached lower values than *M. isabellina* (0.12 and 0.18 g/g in the GH and SS, respectively). *T. elegans* showed by far lower yield values than the other strains (a maximum of 0.06 g/g in the GH/N1).

The oil productivity (g oil/L/day) in the cultures was significantly higher in the SS compared to the GH with *M. isabellina* and *C. echinulata* (Figure 3). *M. isabellina* responded to the N supplementation level, reaching the highest productivity with the SS/N2 (1.31 g/L/day), while in the GH, it reached 1.00 g/L/day at the same N level.

With *C. echinulata* and *T. elegans*, the productivity was significantly lower than with *M. isabellina*, and it was completely unaffected by the N level in both substrates. *C. echinulata* reached a maximum of 0.38 and 0.17 g/L/day in the SS/N3 and GH/N3, respectively. The oil productivity in the *T. elegans* cultures was always below 0.09 g/L/day (Figure 3).

Finally, Nitrogen supplementation and Substrate did not significantly affect the GLA concentration in the cultures, except for *C. echinulata*, which showed the highest mean values in the SS (595 compared to 345 mg/L in the GH), on average, for the N levels. *M. isabellina* and *T. elegans* showed significantly lower values ranging from 83–196 mg/L (Table 6).

In conclusion, *M. isabellina* and *T. elegans* showed, respectively, the highest and lowest oil yield and productivity in the GH and SS. *M. isabellina* and *C. echinulata* showed lower values in the GH than in the SS. Nitrogen tended to decrease the oil yield and increase the productivity, especially with *M. isabellina*. *C. echinulata* was the best GLA producer.

3.5. Prediction of Biodiesel Parameters

Several biodiesel parameters were estimated based on the FAME profile, as reported in Table 7.

All the oil parameters fell within the limits of the U.S. biodiesel standards ASTM D6751 for the OS, KV and CN [38]. The oils from *M. isabellina* only respected the limits for the IV, OS, D, KV, CN, and C18:3, as requested by the E.U. regulations (EN 14214, [39]). *T. elegans* and *C. echinulata* oils exceeded the E.U. limits fixed for the OS and C18:3 contents. Moreover, in some cases, they also exceeded the IV limit and failed to reach the minimum CN value (Table 7).

To determine the effects of the different media, the coefficient of variation (CV%) was calculated on the average of the six media within each strain.

Some parameters showed CV values >5%, i.e., the IV, DU, OS, LCSF, and C18:3, while the other parameters appeared almost unaffected by the media, i.e., the SV, CFPP, HHV, D, KV, and CN (Table 7)

Notably, the *M. isabellina* oils displayed CFPP values near or below 0 °C and reached the highest CN values of up to 61.2 on the GH/N3 (Table 7).

Table 7. Prediction of the biodiesel parameters based on the fatty acid profile of the oils obtained with the different fungal strains cultivated for 6–14 days at 28 °C in shake flasks containing giant reed hydrolysate-based-media (GH) or semi-synthetic media (SS) and 3 increasing levels of nitrogen supplementation (N1, N2, and N3).

Strain	Medium Id	IV	SV	DU	OS	LCSF	CFPP	HHV	D	KV	CN	C18:3
		g I_2/100 g	mg KOH/g	%	h	%	°C	MJ/kg	g/cm^3	mm^2/s	min	%
Mortierella isabellina	GH/N1	88.4	193.8	98.2	8.1	3.7	−3.8	39.8	0.875	4.31	58.6	4.2
	GH/N2	82.8	193.8	91.9	9.1	4.7	−0.4	39.8	0.875	4.36	59.9	4.1
	GH/N3	76.6	194.1	85.0	10.7	5.3	1.8	39.8	0.874	4.40	61.2	3.9
	SS/N1	84.8	193.8	94.4	9.1	3.9	−3.3	39.8	0.875	4.34	59.4	4.2
	SS/N2	83.7	193.9	93.2	9.2	4.0	−2.8	39.8	0.875	4.34	59.6	3.9
	SS/N3	79.7	194.2	88.7	9.9	4.4	−1.5	39.8	0.874	4.36	60.4	3.8
	MEAN	82.7	193.9	91.9	9.2	4.3	−1.7	39.8	0.875	4.35	59.8	4.0
	CV%	5	0	5	10	14	1 [a]	0	0	1	2	4
Cunninghamella echinulata (CCF 3882)	GH/N1	109.0	192.4	112.3	6.1	6.0	3.9	39.8	0.878	4.21	54.5	13.6
	GH/N2	119.0	192.1	119.3	5.5	6.7	6.4	39.7	0.879	4.14	52.4	19.3
	GH/N3	129.0	192.1	128.9	5.1	5.1	0.9	39.7	0.881	4.05	50.0	18.9
	SS/N1	108.6	192.3	113.3	6.3	4.4	−1.4	39.8	0.878	4.21	54.6	12.8
	SS/N2	107.8	192.2	111.5	6.4	5.1	0.8	39.8	0.878	4.23	54.8	12.9
	SS/N3	108.5	192.1	110.6	6.3	5.8	3.5	39.8	0.878	4.22	54.7	13.6
	MEAN	113.6	192.2	116.0	5.9	5.5	2.4	39.8	0.879	4.18	53.5	15.2
	CV%	8	0	6	9	15	1	0	0	2	4	20
Thamnidium elegans (CCF 1456)	GH/N1	112.9	194.5	110.7	5.6	6.1	4.2	39.7	0.879	4.07	52.7	20.0
	GH/N2	127.0	193.4	119.6	5.3	7.4	8.7	39.7	0.881	4.01	50.2	27.3
	GH/N3	126.2	191.8	121.2	5.5	7.5	9.0	39.7	0.880	4.09	51.0	26.8
	SS/N1	101.8	195.8	100.9	6.1	6.5	5.6	39.7	0.877	4.10	54.8	17.2
	SS/N2	112.8	194.7	109.0	5.8	6.5	5.7	39.7	0.878	4.06	52.7	20.7
	SS/N3	123.5	192.6	119.2	5.5	7.9	10.5	39.7	0.880	4.08	51.3	24.7
	MEAN	117.3	193.8	113.4	5.6	6.9	7.2	39.7	0.879	4.07	52.1	22.8
	CV%	8	1	7	5	10	1	0	0	1	3	18
U.S. (ASTM D6751)					>3					1.9–6.0	>47	
E.U. (EN 14214)		<120			>8				0.86–0.9	3.5–5.0	>51	<12

IV = iodine value; SV = saponification value; DU = degree of unsaturation; OS = oxidative stability; LCSF = long-chain saturation factor; CFPP = cold filter plugging point; HHV = higher heating value; D = density; KV = kinematic viscosity; CN = cetane number; and C18:3 = γ-linolenic and α- linolenic acid. The IV and SV were calculated according to Kalayasiri et al. [33]; the DU, LCSF, and CFPP were calculated according to Ramos et al. [34]; the OS was calculated according to Park et al. [35]; the HHV, D KV, and CN were calculated according to Ramirez et al. [36]; [a] was calculated after conversion into Kelvin degrees; ASTM D6751 and EN 14214 are the biodiesel fuels (B 100) standards for U.S. and E.U., respectively [38,39].

4. Discussion

4.1. Sugar Content of the Hydrolysate and Sugar Consumption of the Fungal Cultures

The glucose and xylose contents observed in the hydrolysate (62% glucose and 33% xylose) were roughly proportional to the cellulose and hemicellulose contents in the raw giant reed biomass (41.2% and 22.5%, respectively [13]), indicating that a large fraction of the hemicellulose was retained after the pre-treatment. Other authors, when using an alkali concentration (0.3 M NaOH) similar to that used in our study (i.e., 0.3 mol/kg KOH), reported glucose and xylose concentrations of 45.2 and 17.9 g/L, corresponding to 72% glucose in the hydrolysate [40]. The slightly lower glucose content in our hydrolysate can be explained by the comparatively lower initial cellulose/hemicellulose ratio in our material and, possibly, by the higher hemicellulose retention after the pre-treatment in the present study. In fact, after the pre-treatment, the solids were recovered by filtration and without washing, while the recovered solids were washed several times by Lemões et al. [40], and an extensive washing step can determine a higher hemicellulose loss [41], increasing the relative glucose content in the hydrolysate.

The fast sugar consumption by *M. isabellina* observed in the relatively N-rich media was expected, as this fungus rapidly consumed sugars under comparable initial concentrations (e.g., 28.1 g/L glucose or 26.6 g/L xylose) [18]. Consistent with our observation, these authors noted a decrease in the sugar consumption rates at increasing C/N (low N level available).

Regarding *C. echinulata*, the presence of still unconsumed substrate in the condition of low N availability was also observed by other authors, as well as the slower glucose consumption of this species compared to *M. isabellina* [42]. The faster growth of *M. isabellina* compared to *C. echinulata* was already reported [43]. The overlapping trends in the GH and SS media observed for both *C. echinulata* and *M. isabellina* were unexpected because other authors highlighted much lower glucose consumption rates in hydrolysate derived from alkaline pre-treatment compared to similar artificial media. This was due to the presence of inhibitors affecting the expression and activity of the sugar and ion transporters in the cell membrane [18]. However, in our case, it should be noted that a large fraction of the soluble inhibitors was probably eliminated by filtration.

The slow and partial sugar consumption displayed by *T. elegans*, when grown in the GH media under low N conditions, can be explained by the higher sensitivity to inhibitors of this strain compared to the other two species. In fact, a highly variable growth response depending on the fungal species to giant reed alkali extract was previously highlighted [26]. However, *T. elegans*' growth remained unaffected in the polyphenols-rich media derived from olive mill waste, while *C. echinulata* and *M. isabellina* were partially inhibited [44]. It should be also considered that, although phenols usually show the greatest negative effect on fermentation, the degradation products from the chemical pre-treatment of lignocellulosic biomass usually include several classes of inhibitors, like carboxylic acids, furans, and inorganic salts, able to penetrate the cell membranes [18], toward which *T. elegans* could be comparatively more sensitive than the other two species.

4.2. Cell Biomass and Oil Concentrations, and Cellular Oil Content at the End of the Fermentation

Depending on the level of N supplementation, the N content varied in the formulated media, ranging from 0.14 to 0.47 g/L (Table 1) on top of the endogenous N from the potato extract or giant reed hydrolysate (around 0.07 g/L). This allowed us to evaluate the impact of the N level on the fungal growth and lipid accumulation and composition, as well as on the existence of possible tradeoffs for the different strains. It is worth noting that a large fraction of the provided N was organic, since the presence of some nutrients such as amino acids and vitamins may enhance both cell growth and lipid accumulation [2]. The observed increase in the cell biomass concentration along with the increasing N content is consistent with previous observations of yeasts grown in giant reed hydrolysates [13]. Some authors observed a large increase in the cell biomass concentration for *C. echinulata* from low to intermediate N doses with different sources, and a plateau or even a decrease with the highest dose tested (N 1–1.5 g/L with glucose 70 g/L) [45]. Other authors, using glucose at 30 g/L and nitrogen at 0.5 or 1.0 g/L to investigate the effect of N limitation on the growth of *C. echinulata* and *M. isabellina*, noted that the concentration value of the biomass produced was almost unaffected [30]. Overall, these observations, i.e., our results and the literature cited above, indicate a strong response of the cell biomass to the N level at high C/N followed by a plateau.

The cell biomass concentration values obtained here with *M. isabellina* are consistent with those previously reported with a similar glucose content [18]. These authors reported 11.4–13.3 g/L in 28.1–35.4 g/L glucose media. Interestingly, a higher cell biomass concentration (13.75 g/L) for *M. isabellina* grown on 27.2 g/L sugars from dilute acid pre-treated giant reed was previously observed [46].

The higher values of the biomass concentrations in g/L observed for *M. isabellina* compared to *C. echinulata* are consistent with previous observations with comparable sugar content [30] (8–9 and 9–11 g/L, respectively, for *C. echinulata* and *M. isabellina*). A higher cell biomass concentration for *M. isabellina* compared to *T. elegans* was previously reported after growth in 58 g/L of cheese whey lactose [47]. The relatively poor growth observed in our study for *T. elegans* may be partially explained by the scarcity of some limiting nutrients in all the formulated media. In preliminary experiments, the presence of a small amount of potato extract in the SS media strongly enhanced the cell growth and biomass accumulation compared to the SS media without potato extract.

The higher cell biomass concentration values observed in the SS compared to the GH can be explained considering that: (i) glucose can be more efficiently converted compared to sugar mixtures and (ii) the presence of residual inhibitors in the GH may have reduced the cell growth. Consistent with our observations, other authors reported for *M. isabellina* higher cell biomass values after growth in media containing pure glucose compared to xylose as well as in a synthetic hydrolysate compared to a hydrolysate from alkali pre-treated corn stover [18]. Similarly, other authors reported a moderate decrease in the cell biomass concentration and lipid yield in a medium containing sugars from alkali pre-treated corn cobs compared to a synthetic medium with commercial glucose and xylose [19].

Based on the literature, the observed significant effect of the N supplementation level on the oil concentration was expected. A previous study conducted on oleaginous yeasts grown in a giant reed hydrolysate at increasing N supplementation levels reported an increase followed by a plateau or by a significant decrease in the oil concentration [13]. A similar effect of depressing the oil concentration using relatively high N levels was reported in oleaginous *Mucorales* [30].

The maximum oil concentration obtained with *M. isabellina* in giant reed hydrolysate at the lowest N level (5.6 g/L in GH/N1) was comparable to the values observed with the most productive oleaginous yeast out of five tested in similar conditions, i.e., *Cutaneotrichosporon oleaginosum*, yielding 5.6 g/L with 30 g/L sugars from alkali pre-treated giant reed [13]. The values observed here are higher than those reported concerning *M. isabellina* grown in hydrolysates from acid pre-treated giant reed, yielding 3.02 g/L from 27.2 g/L sugars [46]. With initial sugar concentrations in the media like those used in the present study, values of the oil concentration higher than 5.6 g/L are commonly reported in the literature for *M. isabellina* when using purified sugars [18], but when using lignocellulosic hydrolysates or complex mixtures, lower oil concentrations are generally reported. For instance, 3.6 g/L oil were obtained with 25.7 g/L reducing sugars from rice hulls hydrolysate [48]; 2.5 $\pm$ 1.1 g/L with 28.3 g/L sugars from alkali pre-treated corn stover [18]; 3.18, 4.4 and 3.71 g/L with 27–28 g/L sugars from acid pre-treated corn stover, switchgrass, or Miscanthus hydrolysates, respectively [46]; 2.63 g/L oil with 25.6 g/L reducing sugars from wheat straw hydrolysate [49]; and 3.8 g/L oil with 60 g/L sugars from molasses [43]. A higher oil concentration (8.6 g/L oil) was obtained with this fungus grown in 50 g/L sugars from hydrolyzed steam-exploded corn stover [50].

A two-fold higher oil concentration compared to that reported here for *M. isabellina* was also recently reported (11.6 g/L oil vs. 5.6 g/L oil) [19], albeit with a four-fold higher initial sugar concentration (120 g/L sugars from alkali pre-treated corn cobs vs. 30 g/L GH). All things considered, the sugars from alkali pre-treated giant reed appear a very suitable substrate for producing lipids with *M. isabellina*.

Consistent with our results, the lower performance of *C. echinulata* compared to *M. isabellina* was reported with both species cultivated on purified sugars [51], on molasses [43], or after growth on pectin or lactose [30].

The very poorer performance of *T. elegans* compared to *M. isabellina* was already reported: *T. elegans* CCF 1465 yielded 0.7 g/L oil vs. 8.0 g/L oil of *M. isabellina* cultivated in cheese whey lactose 58 g/L [47].

The tendency of the cellular oil content to decrease along with the increasing N supplementation level observed in this study is consistent with a previous study regarding oleaginous yeasts grown on giant reed hydrolysates [13]. Other authors tested *M. isabellina* in rice hull hydrolysates at three C/N ratios and found an increasing fat-free biomass content at an increasing C/N [48].

Values exceeding 60% in terms of the oil content, such as those observed here for *M. isabellina* in the GH, were only reported for media containing pure glucose [43] or xylose, whereas values lower than 60% were generally reported for more complex substrates like molasses (54% lipid content) or wheat straw hydrolysates (39.4% lipid content) [49]. A lipid content in the cell biomass of 50–57% was observed after growing *M. isabellina* in hydrolysates from alkali pre-treated corn cobs [19] or steam-exploded corn stover [50].

The values of the oil content for *C. echinulata* observed here (40.9% and 51.4% in the GH/N1 and SS/N1) fall within the highest range in the context of the previous literature. In fact, for *C. echinulata*, values of the oil content lower than 50% are generally reported: 43–48% after growth in 50 g/L glucose of three different strains [17], and 7.6–39% lipid content after growth of two strains on several media [45], 31–37% lipid content of three *C. echinulata* strains in 50 g/L glucose [51], 53% oil content was reached in xylose [42], and only 25–26% in 100 g/L starch [52].

In conclusion, *M. isabellina* displayed the highest oil content and concentration with low N supplementation, as verified in the pure glucose media (SS), as well as a superior ability to accumulate lipids compared to *C. echinulata* in a lignocellulose-derived hydrolysate like the GH. This latter observation is consistent with previous reports by other authors comparing different strains of *C. echinulata* and *M. isabellina* and obtaining lower oil content values for the former species in different substrates [43].

4.3. Fatty Acid Profile

The FA contents for *M. isabellina* are within the ranges reported by [53] and in very good accordance with those reported previously by [49] in wheat straw hydrolysates, suggesting a quite stable FA profile for this species irrespective of the growth media (commercial glucose or lignocellulosic derived media).

The FA contents for *C. echinulata* (CCF 3882) are consistent with those reported for *C. echinulata* ATHUM 4411 grown in glucose with yeast extract and ammonia as N sources [45,54], whereas they differ significantly from those reported by other authors who reported around 8–10 percentage points higher for the palmitic and oleic acid contents as well as 8–9 percentage points lower for the linoleic and linolenic acid contents [55]. These differences could be explained by the different strains analyzed or, in part, by the different extraction method (supercritical CO_2) utilized by these authors.

The FA contents for *T. elegans* fell within the ranges reported for palmitic palmitoleic oleic and linoleic acid [56,57]; however, we found a higher GLA content (17 vs. 6–10%). No arachidonic acid was found, which is consistent with some authors [56] and different from other authors reporting a significant amount of this FA [57]. However, it should be noted that very high variations in the FA profile were reported for this species depending on the culture age, in particular for C20:4 varying eight-fold (2.9–24%) and C18:3 (trace-10%) [57]. A decrease in the GLA content during lipid accumulation is commonly observed in oleaginous *Zygomycetes* [58,59], since GLA is synthesized mainly during the growth exponential phase when the growing mycelia need GLA to support cellular membrane expansion [60].

Besides the effect of the factor Strain, the observed variations in the GLA content could be linked also to: (i) the sugar composition of the substrates (glucose–xylose mixture in GH vs. pure glucose in SS); (ii) the level of nitrogen; and (iii) the presence of other minor components in the hydrolysate like polyphenols.

The statistically significant effect of the substrate was not observed in previous reports on oleaginous yeasts [13] nor in oleaginous fungi [18,42]. In particular, some authors stated that the SCO produced by *C. echinulata* and *M. isabellina* had a similar composition that was more or less independent of the carbon source utilized (pure glucose or xylose) [18,42]. However, it should be noted that a proper statistical analysis was not provided.

The observed increase in the GLA content in the GH cultures of *C. echinulata* in response to increasing N supplementation is consistent with data reported previously by other authors [42]. In fact, when using pure xylose, these authors reported a slight increase in the GLA content in the cells of *C. echinulata* at 144 h with an increasing N level. In our study, the increasing effect of nitrogen on the GLA in *C. echinulata* did not reach statistical significance within the SS media.

Finally, an effect on the FA profile due to the presence of polyphenolic compounds, as in the GH-based media, cannot be excluded, as it was previously observed for another oleaginous microorganism: a polyphenol-rich aqueous extract of *Prosopis cineraria* has been

demonstrated to alter the FA composition in the oleaginous yeast *Rhodotorula mucilaginosa*; in particular, it increased the linolenic acid [61].

Interestingly, Yu et al. [62], reported very similar FA profiles for *C. oleaginosum* grown on pure glucose or xylose but diverging FA profiles when adding pre-treatment-derived inhibitors. Thus, in our study, xylose in combination with the other GH minor components (like polyphenols) may have played a role in affecting the FA profile.

4.4. Oil Yield and Productivity and γ-Linolenic Acid Concentration

As far as the oil yield per gram of sugar is concerned, it should be considered that while the maximum theoretical glucose to lipid yield is 0.31 g/g of sugar, documented conversion yields above 0.20 g/g are rare [18]. This suggests the existence of some limiting factor associated with this threshold for lipid accumulation from glucose [42]. Nitrogen supplementation affected the oil yield, as the highest values were obtained for *M. isabellina* and *C. echinulata* (0.23 and 0.18 g/g, respectively) in the SS/N1 containing pure glucose and the lowest N level. The lower values obtained in the GH (0.19 and 0.12 g/g for *M. isabellina* and *C. echinulata*, respectively) compared to the SS can be explained by the presence of inhibitors or by a lower conversion yield from the xylose in the hydrolysate as it can be metabolized through the pentose phosphate pathway [63], which is less efficient than glucose metabolism for lipid production [18,49]. Regarding *M. isabellina*, the yield obtained in the GH was higher than that observed by other authors in pure glucose or hydrolysates from corn stover or wheat straw (0.14 and 0.123 g/g, respectively) [18,49]. This could be explained by the lower toxicity of the GH due to the filtration of the pre-treated biomass before the enzymatic hydrolysis [13] and by the presence of other non-sugar nutrients. For example, *M. isabellina* may have benefitted from the presence of acetate released by the pre-treatment [64], since it can consume acetic acid and glucose simultaneously [18]. In addition, acetic acid not only participates in cell mass growth but may also contribute to microbial lipid accumulation [65,66]. Higher conversion yields were also reported for *M. isabellina* grown in the hydrolysate of SPORL pre-treated Douglas fir (0.21 g/g) [67], rice hull hydrolysate (0.215 g/g) or sweet sorghum extract (0.242 g/g) [48].

In our study, *C. echinulata* reached lower conversion yield values than *M. isabellina*, suggesting a higher sensitivity to inhibitors in comparison to *M. isabellina*. In general, a reduced yield in hydrolysates from the lignocellulosic biomass compared to the semi-synthetic media containing glucose was not unexpected, as it was already reported for several oleaginous strains [49]. A comparable yield value (0.13 g/g) can be calculated from data reported about the cultivation of *C. echinulata* in 60 g/L sugars from molasses [43].

In terms of oil productivity, the higher oil productivity in the SS compared to the GH observed for *M. isabellina* and *C. echinulata* (1.31 vs. 1.00 g oil/L/day, respectively) was expected, since higher values are generally observed in glucose compared to more complex sugar sources [18,43]. The values observed here for *M. isabellina* in glucose (0.87–1.31 g/L/day) are consistent with the values reported in the literature for this species, ranging 0.79–1.73 g/L/day [42,43,46,68]. For this strain, an effect of the factor nitrogen was highlighted, as we observed an increase in the oil productivity values in the SS with the highest N supplementation, consistent with the literature, which reported increasing oil productivity in glucose along with decreasing C/N values [18]. Overall, GH after alkali pre-treatment appears suitable for obtaining a high productivity level, reaching 1.00 g oil/L/day. This value has to be compared to 0.5 g L/day for *M. isabellina* in wheat straw hydrolysates [49] or to 0.76–0.90 g/L/day for the same species grown in dilute acid hydrolysates of corn stover, switchgrass and miscanthus [46]. Notably, the productivity values observed here for the GH after alkali pre-treatment are almost double compared to those relative to hydrolysate from dilute acid pre-treated giant reed (0.53 g/L/day) [46] or to molasses calculated from data reported by other authors for both *M. isabellina* and *C. echinulata* (0.82 and 0.27 g/L/day, respectively) [43]. Similar productivity values (0.93–1.07 g/L/day) were reported for *M. isabellina* grown on sugars from hydrolysates from steam-exploded corn stover [50]. The relatively poorer performance of *T. elegans*

compared to *M. isabellina* is consistent with what was already reported by other authors, who reported a productivity of 0.7 g/L in 220 h corresponding to 0.08 g/L/day [47].

Regarding the potential oil production per hectare, giant reed is considered more recalcitrant to bioconversion compared to other lignocellulosic feedstocks like switchgrass or miscanthus [10], yielding 355–489 g of sugars per kg of dry biomass [13,23,40,46]. Estimations based on the oil yield of *M. isabellina* in this work (Figure 3) suggest that up to 93 kg of oil could be produced by fungi per Mg of giant reed dry raw biomass. This value has to be compared to 103 kg lipid per Mg of wheat straw [49]; however, giant reed has outstanding dry matter productivity, reaching up to 59 Mg/ha per year under favorable environmental conditions [4]. Thus, up to 5.5 Mg oil per hectare per year could be achievable with *M. isabellina*.

Concerning the γ-linolenic acid concentration, nitrogen supplementation did not affect the GLA concentrations in the cultures at all. The substrate type only affected this variable in the case of *C. echinulata*, which provided substantial GLA production in the SS (595 mg/L compared to 345 mg/L in the GH, on average, given the N levels), considering the amount of sugar provided to the cultures. These values are broadly consistent with the values reported by other authors who obtained a maximum yield of 396 mg/L with *C. echinulata* ATHUM 4411 after 12 days of cultivation with tomato waste hydrolysate as the N source in fed-batch with 10 plus 30 g/L glucose [45]. These authors reported significantly lower values when using N sources different from tomato waste in media containing 70 g/L glucose (274, 282, and 326 mg/L with corn steep, whey concentrate, and yeast extract, respectively). Since we observed the best results in the SS media containing potato extract, it could be hypothesized that compounds from fresh *Solanaceae* could promote the GLA production by *C. echinulata*. Contrary to our results, these authors reported GLA production with a strong response to the N dose, with up to two- to three-fold variations (although no statistical analysis was provided).

Even higher GLA concentrations were reported in the literature with another *C. echinulata* strain (CCRC 31840), yielding 964–1349 mg/L when grown on starch [52,69] and up to 742 g/L with *C. echinulata* ATHUM 4411 after 309 h in commercial glucose [43]; however, it should be noted that these concentrations were obtained with media with significantly higher carbohydrate contents (100 g/L starch, 60 g/L glucose) compared to the 30 g/L sugars in our experiments. Thus, the GLA concentrations in our experiments, if calculated per gram of carbohydrates, are consistent with those in previous reports. The higher concentrations observed when using commercial glucose (SS) compared to a more complex sugar source (GH) are consistent with previous observations by other authors who obtained higher (up to three-fold) GLA concentrations when using pure glucose compared to carbohydrates from molasses [43].

The comparatively lower values observed here with *M. isabellina* (166–196 mg/L) were consistent with previous reports [43,70], while the GLA production by *T. elegans* (83 mg/L) was unsatisfactory even accounting for the lower substrate concentration used here (30 g/L sugars), as the strain was reported to reach up to 664 mg/L in 100 g/L glycerol [56]. The same strain was reported to yield up to 3.5 g/kg of moist substrate (apple pomace impregnated with peanut oil) in solid-state fermentation [71], suggesting the poorer performance of the strain under agitation (as in this study).

4.5. Assessment of Biodiesel Parameters

In general, most of the calculated biodiesel properties for the GH are similar to those obtained for the SS media— namely, the SV, CFPP, HHV, D, KV, and CN—suggesting that the lipids obtained from giant reed hydrolysates could be a suitable feedstock for biodiesel preparation, with different properties depending mostly on the strain.

All the oils from *T. elegans* (CCF 1456), *C. echinulata* (CCF 3882), and *M. isabellina* were within the limits of the U.S. biodiesel standards (ASTM D6751). However, only the oils from *M. isabellina* were suitable for biodiesel production according to the more stringent E.U. regulations (EN 14214).

Interestingly, the *M. isabellina* oils reached estimated CN values of up to 61.2 in the GH/N3, which were higher than the values reported for several vegetable oils or animal fats [36] and comparable to those reported for *Mucor plumbeus* and *Aspergillus oryzae*, for the yeast *C. oleaginosum*, or for palm oil-derived biodiesel [72]. Estimated CN values from *M. isabellina* oils exceeding 56 [19,73] and up to 60.9 [49] were previously reported. The high CN value of *M. isabellina* indicates good combustibility even compared to D2 petroleum diesel [74]. The HHV values were also similar to those of biodiesel from several vegetable oils or animal fats [75] and in very good accordance with the values (39.7–39.8 MJ/kg) reported for *M. isabellina* by other authors [49]. The viscosity and density were also almost identical to a previous report [49]; however, in that work, lower IV were reported (72–78) compared to 82.7, on average, in this work. Notably, the CFPP values were near or below 0 °C for *M. isabellina*, indicating good low-temperature fluidity. Although this parameter is not highlighted nor regulated by the U.S. or E.U. biodiesel standards [38,39], the application of this kind of biodiesel could be optimal in colder climates or seasons.

5. Conclusions

This study demonstrated that it is possible to achieve a remarkable production of microbial oil utilizing giant reed biomass as high-yield, low-cost, renewable feedstock. The necessary pre-treatment of the dried lignocellulosic biomass with KOH at 120 °C was effective in producing enzymatic hydrolysates rich in glucose and xylose, which resulted in suitable fermentation substrates for the selected oleaginous fungal strains. To the best of our knowledge, this is the first study reporting data about the bioconversion of giant reed biomass into lipids by *M. isabellina*, *C. echinulata* (CCF 3882), and *T. elegans* (CCF 1456).

Among the strains, *M. isabellina* showed outstanding values of the final cell and oil concentrations (10.9 and 5.6 g/L, respectively, from media with 30 g/L sugars), as well as of the oil content, oil yield, and oil productivity (63.4%, 0.19 g/g, and 1 g/L/day, respectively), in the GH. These values were often lower than those observed with pure glucose (SS media), probably due to the lower conversion efficiency of xylose compared to glucose and/or the presence of some pre-treatment-derived inhibitors in the hydrolysates.

The level of N supplementation in the formulated media affected the oil production: a low N level favored the cellular oil concentration and content and the oil yield per gram of sugar consumed, while higher N levels increased the daily productivity. The FA profile was mainly determined by the strain. Interestingly, the N level in some cases affected the intracellular GLA content but never affected the GLA final concentration in the cultures.

Among the strains, *C. echinulata* appeared the most interesting GLA producer, achieving 345 mg/L of this FA in the GH, even if much higher concentrations were achieved in pure glucose, while *M. isabellina* appeared very well suited for biodiesel production from giant reed hydrolysate. In fact, the *M. isabellina* oils were always rich in oleic and palmitic acid and low in PUFA, and most of the estimated biodiesel parameters were almost unaffected by the different fermentation substrates.

Further studies are envisaged to maximize the oil production also by inoculum/feeding strategy optimization and media formulation.

From an economic perspective, the formation of multiple products in a biorefinery cycle would make the process more profitable. Thus, the discarded fractions after pre-treatment and oil extraction (i.e., lignin-rich liquefied biomass, undigested fibers, and defatted fungal biomass) could be recovered and valorized to obtain, for instance, biomethane or other bio-products with higher added value, like high-quality chitin, to be used in medicine or pharmacology. Future works will consider a detailed economic study to assess the economic viability of the proposed method.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/en16145398/s1, Table S1: List of abbreviations.

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