processes

MDPI

Article

New Insights on Protein Recovery from Olive Oil Mill Wastewater through Bioconversion with Edible Filamentous Fungi

Taner Sar [1,2], Murat Ozturk [2], Mohammad J. Taherzadeh [1] and Jorge A. Ferreira [1,*]

[1] Swedish Centre for Resource Recovery, University of Borås, 50190 Borås, Sweden; st.taner@gmail.com (T.S.); mohammad.taherzadeh@hb.se (M.J.T.)

[2] Department of Molecular Biology and Genetics, Gebze Technical University, Gebze-Kocaeli 41400, Turkey; murat.ozturk@gtu.edu.tr

* Correspondence: jorge.ferreira@hb.se; Tel.: +46-(0)3-3435-4638

Received: 20 August 2020; Accepted: 19 September 2020; Published: 25 September 2020

Abstract: Olive oil mills represent an important sector in the Mediterranean Sea Basin but also an environmental hazard due to untreated wastewater. Recovery of nutrients from olive oil mill wastewater (OMWW) as protein-rich microbial biomass can produce novel feed and reduce its chemical oxygen demand; however, low-protein containing products have been reported. New strategies leading to higher protein-containing fungal biomass could renew the research interest on bioconversion for pollution mitigation of OMWW. In this work, through cultivation of edible filamentous fungi (*Aspergillus oryzae*, *Neurospora intermedia*, and *Rhizopus delemar*), a link between the protein content in the originated fungal biomass, and the addition of nitrogen and medium dilution was established. Addition of nitrogen in the form of $NaNO_3$ reduced the cultivation time from 96 h to 48 h while achieving a similar biomass mass concentration of 8.43 g/L and increased biomass protein content, from w = 15.9% to w = 29.5%. Nitrogen addition and dilution of OMWW, and consequent reduction of suspended solids, led to an increase in the protein content to up to w = 44.9%. To the best of our knowledge, the protein contents achieved are the highest reported to date and can open new research avenues towards bioconversion of OMWW using edible filamentous fungi.

Keywords: *Aspergillus oryzae*; bioconversion; feed proteins; olive oil mills; olive oil mill wastewater; pollution mitigation; wastewater treatment

1. Introduction

Olive oil production is an important economic sector in the Mediterranean Sea Basin. Olive oil is extracted through the so-called discontinuous (pressing) or continuous (centrifugation) processes. The pressing process is the oldest method and benefits from cheap equipment, technical simplicity, and low generation of wastewater, but it is labor-intensive. The centrifugation process is an automated process and more common within the sector; however, it produces a large amount of wastewater [1]. In this process, olives are washed and crushed, and then mixed in a tank for a certain period of time to obtain oil droplets. This is followed by a series of centrifugation steps to achieve olive oil separation. Concomitantly, large amounts of byproducts, namely, pomace and olive oil mill wastewater (OMWW), are generated (Figure 1).

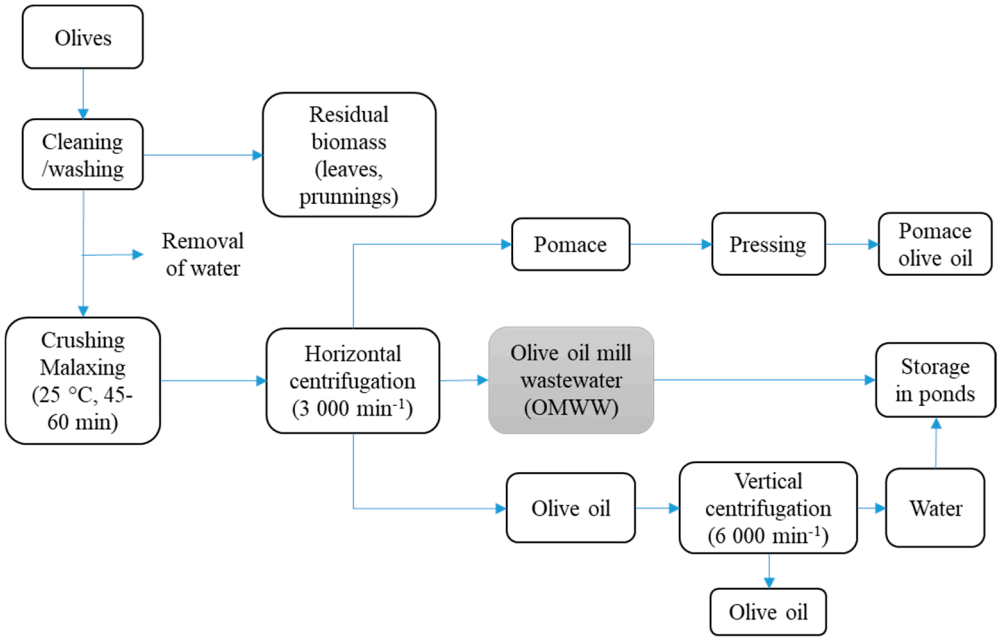

Figure 1. Overview of the main steps taking place in an olive oil mill, adopting the continuous (centrifugation) process. The production of olive oil mill wastewater (OMWW), presently left untreated and thereby with high environmental pollution potential, is highlighted.

OMWW represents an environmental hazard in view of its slightly acidic pH, high content of toxic compounds such as phenols, and high chemical oxygen demand (COD) [2,3]. Moreover, olive oil production is seasonal and connected to the harvest period, which influences the range of treatment strategies that can potentially be applied. If left untreated, OMWW can affect the color of natural waters and create odor and pollution due to emission of methane and hydrogen sulfide [4]. Consequently, OMWW is considered a potential contaminant source for flora and fauna associated with soil and water pollution [3]. To overcome its environmental hazard, several strategies, including aerobic and anaerobic biological processes, have been applied to reduce the polluting load of OMWW [1,5]. Alternatively, research approaches have also attempted to extract value from OMWW. For instance, extraction of phenolic compounds, such as hydroxytyrosol and tyrosol, with potential use as antimicrobial, antioxidant, and anti-inflammatory agents, has been reported [6,7]. Due to its composition, rich in, e.g., sugars, fat, and polyalcohols, OMWW has also been considered a potential substrate for production of hydrogen [8], biofuel through supercritical water gasification [9], enzymes [10–12], and protein-rich microbial biomass [13].

The use of microorganisms, especially edible filamentous fungi, for the valorization of industrial wastewaters and other low-value byproducts, has been among the most studied strategies. In addition to the wide range of enzymes, enabling growth in most (if not all) substrates, and value-added products that can be produced, the macroscopic mycelium, obtained following assimilation of nutrients and easily recovered from the medium, provides superior performance of filamentous fungi towards reduction of COD levels, in comparison to unicellular microorganisms (e.g., bacteria, yeasts, and microalgae) [14]. The use of the latter normally entails costly biomass recovery processes. Filamentous fungal biomass normally contains high protein levels, essential amino acids, polyunsaturated fatty acids, and its cell wall is rich in compounds with immunostimulant activity [15]. Therefore, it has increasingly been studied as a replacement of fish meal and soybeans in the production of animal feed, a product in increasing demand due to population growth. The growth of filamentous fungi in other fat-rich substrates such as dairy products [16–18] and fish processing-derived wastewaters [19,20], for protein recovery, has been reported in the literature. Research studies on protein recovery from OMWW using filamentous fungi, although scarce and more than eight years old, also exist [21–23]. However, these are

characterized by low protein-containing products if the landscape of protein contents reported among fungal biomasses obtained from the growth in various low-value substrates is considered.

The aim of the present study was to develop a new cultivation strategy to enhance the protein level present in fungal biomass obtained from the cultivation of edible filamentous fungi in OMWW. Through nitrogen addition, and nitrogen addition and medium dilution, to lower the content of suspended solids, the protein content of *Aspergillus oryzae* biomass grown in OMWW, could be increased from w = 15.9% to w = 32.3% and w = 44.9%, respectively.

2. Materials and Methods

2.1. Filamentous Fungal Strains

Three edible filamentous fungi were used in this work, namely, *Aspergillus oryzae* var. *oryzae* CBS 819.72, isolated from tané koji used for sake making, *Neurospora intermedia* CBS 131.92, isolated from oncom, and *Rhizopus delemar* CBS 145940, isolated from leaves used in tempeh production (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands). The strains were maintained on PDA (Potato Dextrose Agar) medium plates containing 4 g/L potato infusion, 20 g/L glucose, and 15 g/L agar. New plates were prepared every 2–4 weeks via addition of 20 mL of sterile distilled water to pre-grown plates, where the spores were brought into solution using a L-shape sterile disposable plastic spreader. New plates were inoculated with 100 µL of spore solution, which was evenly distributed onto the agar surface with another plastic spreader, followed by incubation at 30 °C for three days and then stored at 4 °C until use.

2.2. Substrate

The olive oil mill wastewater (OMWW) was obtained from Dizem Olive Oil Factory (Çanakkale, Turkey). The steps leading to the production of OMWW are presented in Figure 1 and its composition provided in Table 1. The OMWW was heat-sterilized in an autoclave (VX-95, Systec, Linden, Germany) at 121 °C for 20 min, and then stored at 4 °C until use.

Table 1. Characterization of OMWW used in the study.

Parameter	Value
pH	5.2 ± 0.1
Total COD (g/L)	102.50 ± 4.24
Dissolved COD (g/L)	54.50 ± 1.41
Total solids (g/L)	65.23 ± 0.14
Dissolved solids (g/L)	35.09 ± 1.60
Volatile solids (g/L)	52.79 ± 1.39
Ash (g/L)	13.15 ± 0.83
Nitrogen (g/L)	1.32 ± 0.00
C/N ratio [1]	22.21
Protein (g/L)	8.26 ± 0.03
Fat (g/L)	16.73 ± 0.63
Glucose (g/L)	7.69 ± 0.38
Other sugars (g/L)	2.78 ± 0.08
Glycerol (g/L)	1.19 ± 0.57
Lactic acid (g/L)	0.37 ± 0.00
Acetic acid (g/L)	0.75 ± 0.06
Ethanol (g/L)	1.77 ± 0.09

[1] Calculated by considering the nitrogen content and assuming volatile solids being composed of 55% carbon [24].

2.3. Cultivation in Shake-Flasks

Series of cultivations were carried out in 250 mL wide-neck Erlenmeyer flasks containing 50 mL of OMWW. The factors studied included fungal strain, initial pH (pH 5.2 vs. pH 6.5 adjusted with 2 mol/L

NaOH, incubation time (varied within 24–120 h), OMWW dilution (diluted with ultrapure water to make up (20, 40, 60, and 80) % OMWW), nitrogen supplementation using $NaNO_3$ and NH_4Cl, $NaNO_3$ addition mass concentration, and OMWW fraction. For the latter, OMWW was centrifuged (Heraeus Megafuge 8, Thermo Scientific, Waltham, MA, USA) at 4000× *g* for 10 min; the supernatant was used directly for fungal cultivation, while the same amount of ultrapure, as that of supernatant, was added to the wet cake, preceding pH adjustment to 5.2, sterilization, and fungal cultivation. Each flask was inoculated with 1 mL of spore solution, prepared as aforementioned, followed by incubation in water baths (Excella E24, New Brunswick Scientific, Nürtingen, Germany) at 35 °C with rotational frequency of 125 min^{-1}. No pH control and readjustment was carried out during cultivation. After the cultivation, the originated cultures were poured into a sieve (1 mm^2 pore area), and the recovered fungal biomass was thoroughly washed with distilled water for further oven drying. All cultivations were carried out in duplicate.

2.4. Analytical Methods

The harvested biomass samples were dried to constant weight in an oven at 70 °C. Total solids (TS), suspended solids (SS), dissolved solids (DS), and ash contents were determined according to Sluiter et al. [25] and Sluiter et al. [26]. Crude protein content of OMWW and fungal biomass were analyzed according to the Kjeldahl method [27]. To determine the crude protein content of the samples, a nitrogen-to-protein conversion factor of 6.25 was used [28]. The COD levels of both OMWW and the liquids remaining after fungal cultivation and fungal biomass separation were determined using a COD Kit (COD 1500, Nanocolor, Düren, Germany). Total fat contents were determined based on diethyl ether extraction method described in a previous work [19]. Alkali-insoluble material, representing the fungal cell wall fraction, was quantified according to a previously published method [29]. Sugars, organic acids, and ethanol levels were monitored using high-pressure liquid chromatography (HPLC) (Waters 2695, Waters Corporation, Milford, MA, USA) equipped with an analytical ion exchange column based on hydrogen ions (Aminex HPX-87H, BioRad, Hercules, CA, USA), operating at 60 °C and using 0.6 mL/min of 0.005 mol/L H_2SO_4 as eluent, and a refractive index (RI) detector (Waters 2414, Waters Corporation, Milford, MA, USA).

2.5. Statistical Analysis

The software Minitab17® (Minitab Ltd., Coventry, UK) was used for the statistical analysis of the obtained results with ANOVA (analysis of variance) tables using general linear models. Pairwise comparisons among groups of data were also carried out according to the Tukey test. Significant differences were considered at p-value < 0.05 within a 95% confidence interval. All error bars and intervals presented represent two times the standard deviation.

3. Results

3.1. Composition of Olive Oil Mill Wastewater (OMWW)

The chemical composition of OMWW is presented in Table 1. OMWW composition may vary according to olive type, season, and olive processing method [1]. The COD of the OMWW used in this work was found to be 102.5 g/L, being within the range, of 30–320 g/L, reported in the literature [1,5]. Fat, protein, sugars (mainly glucose), and ash were found to be the main compounds present in OMWW, where a C/N ratio of 22.21 was determined. Altogether, at a first glance, OMWW contained all needed nutrients to support fungal growth.

3.2. Production of Fungal Biomass

In the first approach, the three fungal strains were cultivated in undiluted OMWW for 96 h (Figure 2). *A. oryzae* was found to lead to the highest biomass mass concentration, namely of 8.1 g/L, in comparison to that achieved with *Neurospora intermedia* (2.1 g/L) and *Rhizopus delemar* (0.49 g/L). Therefore, further studies,

namely, at varying initial pH using undiluted OMWW (Figure 3a,b) and at varying initial pH and varying OMWW mass concentration (Figure 3c,d), were carried out using only *A. oryzae.* The cultivation of the ascomycete fungus in undiluted OMWW at varying initial pH (5.2 vs. 6.5) led to similar maximum biomass mass concentrations after 96 h of cultivation, where the initial pH generally did not influence the fungal growth rate (except when comparing the biomass mass concentration at 72 h of cultivation where statistically significant differences were found). However, the initial pH was found to play a role on final biomass mass concentration obtained after 96 h of cultivation at varying OMWW mass concentrations, where significantly higher biomass mass concentrations at 40% and 80% OMWW were obtained when the initial pH was 5.2. As it is presented in Figure 3c,d, gradually higher mass concentrations of OMWW did not impair the growth of *A. oryzae*. The biomass mass concentrations based on undiluted OMWW at 20%, 40%, 60%, 80%, and 100% were 8.55 ± 1.03 g/L, 9.33 ± 1.55 g/L, 7.95 ± 0.21 g/L, 9.88 ± 0.30 g/L, and 8.46 ± 0.51 g/L, respectively, which corroborates lack of impairment of fungal growth. Overall, the results show that neither chemicals, for pH adjustment, nor dilution, are needed while attaining the same cultivation output. Therefore, further studies were conducted using undiluted OMWW at its natural initial pH of 5.2.

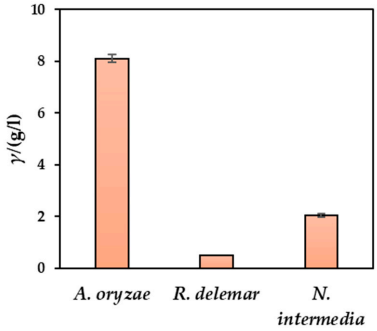

Figure 2. Comparison of the growth performance of *A. oryzae*, *R. delemar*, and *N. intermedia* considering the mass concentration of fungal biomass obtained after 96 h cultivation in undiluted olive oil mill wastewater (OMWW).

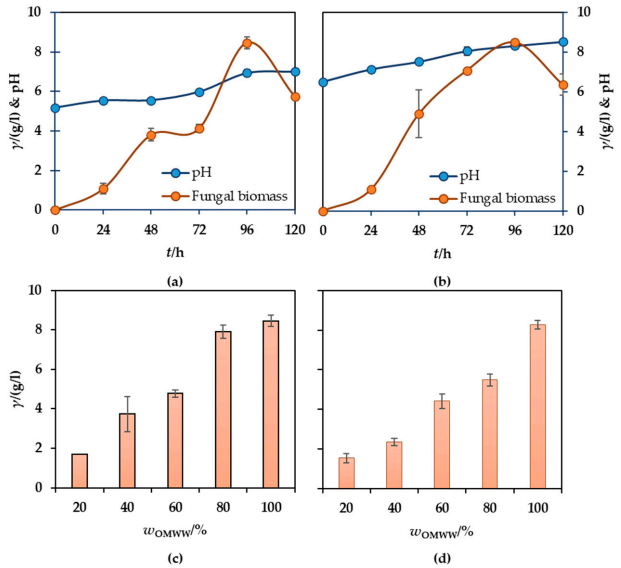

Figure 3. (**a**) Profiles of biomass mass concentration and pH during cultivation of *A. oryzae* in undiluted OMWW at initial pH of 5.2; (**b**) Profiles of biomass mass concentration and pH during cultivation of *A. oryzae* in undiluted OMWW at initial pH of 6.5; (**c**) Fungal biomass mass concentration following 96 h cultivation of *A. oryzae* in different mass concentrations of OMWW at initial pH of 5.2; (**d**) Fungal biomass mass concentration following 96 h cultivation of *A. oryzae* in different mass concentrations of OMWW at initial pH of 6.5.

A previous study has shown that the addition of nitrogen, in the form of NH_4Cl and $NaNO_3$, to OMWW could increase the activity of lipase produced by *A. oryzae* strains [12]. Therefore, a similar strategy was used in this study (Figure 4), and its effect on biomass mass concentration and growth rate investigated. In the first approach, the initial nitrogen amount in OMWW of 1.32 g/L was doubled by addition of $NaNO_3$ and NH_4Cl, representing the addition of 8.01 g/L and 5.04 g/L, respectively. Following a 48 h cultivation, the addition of $NaNO_3$ led to a significant increase in the mass concentration of biomass in comparison to both control cultivation and that where NH_4Cl was added (Figure 4a). A further investigation was carried on the effect of $NaNO_3$ addition to reach a C/N ratio of 11.11 (8.01 g/L) and 5.55 (32.06 g/L), on the biomass mass concentration obtained following cultivation at 24, 48, and 96 h (Figure 4b). It was found that a C/N ratio of 11.11 was preferable for growth of *A. oryzae* in OMWW, which led to a reduction of the cultivation time needed from 96 h to 48 h, while achieving a similar maximum biomass mass concentration. The yield of biomass based on consumed COD was 0.19 g/g.

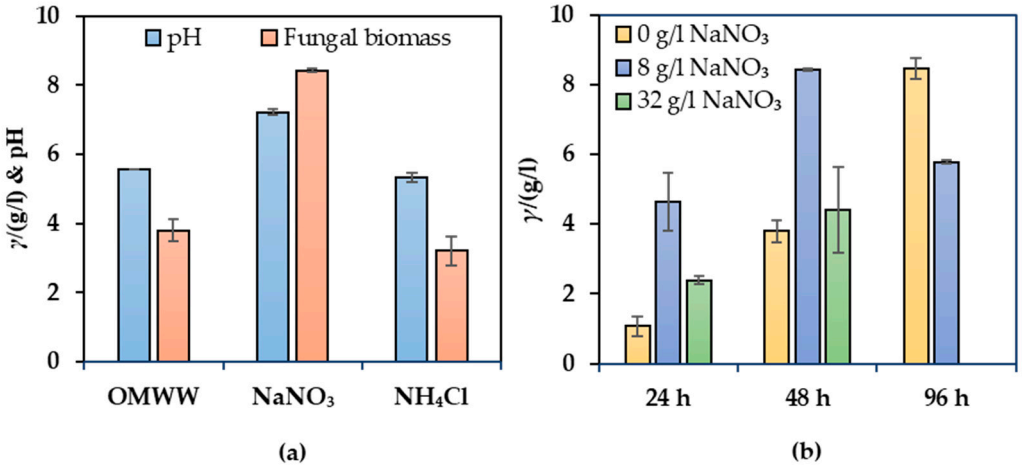

Figure 4. (**a**) Effect of nitrogen source on biomass mass concentration and pH following 48 h cultivation of *A. oryzae* in undiluted OMWW at initial pH of 5.2; (**b**) Effect of mass concentration of $NaNO_3$ on biomass mass concentration following cultivation of *A. oryzae* in undiluted OMWW at initial pH of 5.2 for 24 h, 48 h, and 96 h.

3.3. Composition of Fungal Biomass

In addition to the impact on fungal biomass production, the effect of different cultivation factors on fungal biomass composition was concomitantly monitored. While varying the initial pH (Figure 5a,b), cultivation time (Figure 5a), and mass concentration of OMWW (Figure 5b), the protein content of the harvested biomass was w = 13.3–19.2%. However, when nitrogen was added to the medium (Figure 5c), fungal biomass containing up to w = 32.3 % protein was obtained. Therefore, the addition of nitrogen led to a decrease of the needed cultivation time and to an increase of 54% in protein content, in comparison to the condition without nitrogen addition, following a 48 h cultivation.

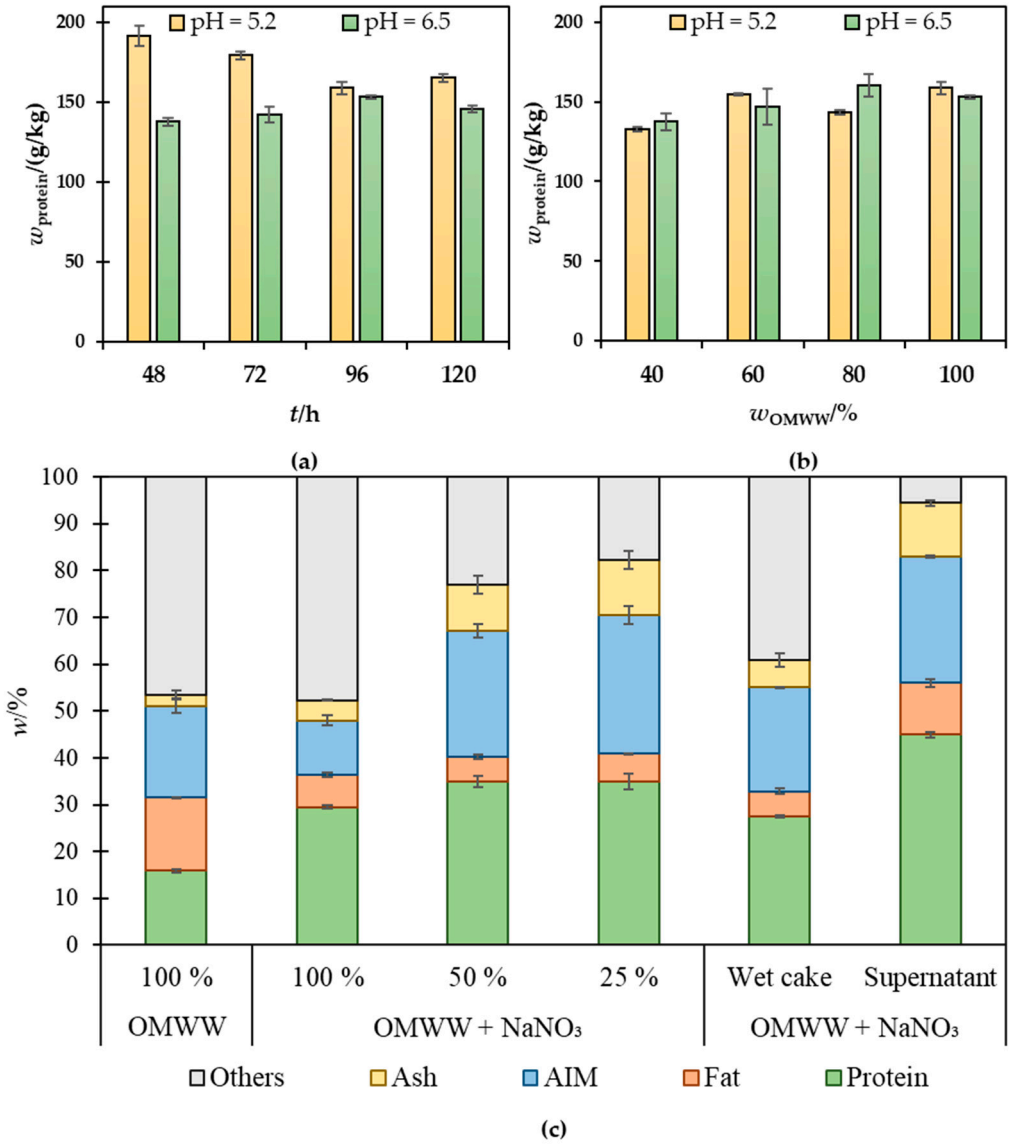

Figure 5. (**a**)Protein content of *A. oryzae* biomass obtained after cultivation in undiluted OMWW at initial pH of 5.2 and 6.5; (**b**) Protein content of *A. oryzae* biomass obtained after cultivation in OMWW at different mass concentrations and at initial pH of 5.2 and 6.5; (**c**) composition of *A. oryzae* biomass obtained at different cultivation conditions.

A more extensive compositional analysis of *A. oryzae* biomass, including fat, cell wall (alkali-insoluble material), and minerals (ash), was carried out and the effect of nitrogen addition, OMWW mass concentration, and presence of suspended solids investigated (Figure 5c). A striking observation is that the amount of fungal biomass that could not be analyzed was the highest when undiluted OMWW or a derived solution based on only suspended solids diluted with ultrapure water, were used. Inversely, a more complete characterization of the biomass was acquired as the level of suspended solids, as a result of OMWW dilution, was carried out, reaching the highest level of characterization when only dissolved solids were used as cultivation medium. The latter cultivation condition also led to the highest protein content obtained in this work, namely, of w = 44.9%. The fungal cultivation in the supernatant obtained following OMWW centrifugation led to the production of 4.23 g/L biomass, while 7.58 g/L of fungal biomass were obtained following cultivation in the wet cake obtained after centrifugation and with added ultrapure water. Yields of fungal biomass per gram of dissolved solids (0.12–0.29 g/g), per gram of total solids (0.13–0.16 g/g), and per liter of undiluted

OMWW (8.43–10.24 g/L) increased as OMWW mass concentration decreased. Altogether, the results of this study point out that optimization of C/N ratio and content of suspended solids can play a substantial role on production of fungal biomass as well as on protein recovery from OMWW.

3.4. Composition of Streams Remaining after Fungal Cultivation and Biomass Harvesting

Due to their growth in the form of a macroscopic filamentous structure easily recoverable from the medium, the use of filamentous fungi for bioconversion of low-value sidestreams can contribute to substantial COD reduction. The characterization of the streams left after 48 h or 96 h fungal cultivation and biomass separation at different conditions is presented in Table 2. The reduction range of total COD was 35–44%.

Table 2. Characterization of the remaining streams following cultivation of *A. oryzae* and biomass separation. The conditions presented are the same as presented in Figure 5c.

Parameter	OMWW (96 h)	OMWW + $NaNO_3$ (48 h)			OMWW + $NaNO_3$ (48 h)	
	100%	100%	50%	25%	Wet Cake	Supernatant
pH	6.93 ± 0.17	7.22 ± 0.08	7.78 ± 0.10	7.91 ± 0.08	7.86 ± 0.04	6.73 ± 0.04
Total COD (g/L)	67.00 ± 2.83	57.50 ± 1.41	24.00 ± 1.41	12.00 ± 0.00	75.00 ± 5.66	58.50 ± 7.07
Dissolved COD (g/L)	46.00 ± 4.24	49.50 ± 4.24	21.25 ± 2.12	10.15 ± 0.99	69.50 ± 4.24	44.50 ± 4.24
Total solids (g/L)	51.50 ± 2.08	59.09 ± 1.42	26.32 ± 0.84	15.05 ± 0.40	14.51 ± 0.09	47.25 ± 4.24
Dissolved solids (g/L)	32.91 ± 3.43	42.00 ± 0.64	18.04 ± 1.40	10.63 ± 1.15	7.08 ± 1.17	34.99 ± 0.49
Volatile solids (g/L)	36.44 ± 0.92	37.99 ± 1.36	11.97 ± 0.05	6.91 ± 0.06	9.91 ± 0.37	31.68 ± 3.18
Ash (g/L)	9.59 ± 1.53	21.52 ± 3.67	14.36 ± 0.76	7.94 ± 0.22	3.95 ± 0.88	15.19 ± 1.32
Nitrogen (g/L)	1.10 ± 0.02	2.25 ± 0.21	2.25 ± 0.21	1.06 ± 0.30	0.70 ± 0.13	1.25 ± 0.06
Protein (g/L)	6.91 ± 0.12	14.07 ± 1.29	14.07 ± 1.29	6.62 ± 1.85	4.39 ± 0.82	7.81 ± 0.39
Glucose (g/L)	1.26 ± 0.20	1.04 ± 0.23	0.50 ± 0.08	0.18 ± 0.14	0.10 ± 0.02	0.99 ± 0.26
Other sugars (g/L)	2.89 ± 0.04	2.63 ± 0.45	1.01 ± 0.06	0.42 ± 0.32	0.27 ± 0.03	2.27 ± 0.69
Glycerol (g/L)	0.61 ± 0.07	0.44 ± 0.08	0.28 ± 0.01	0.13 ± 0.09	0.10 ± 0.03	0.36 ± 0.05
Lactic acid (g/L)	0.22 ± 0.00	0.33 ± 0.07	0.10 ± 0.01	0.24 ± 0.14	0.06 ± 0.01	0.39 ± 0.11
Acetic acid (g/L)	0.67 ± 0.14	0.30 ± 0.08	0.67 ± 0.11	0.37 ± 0.27	0.33 ± 0.12	0.90 ± 0.31
Ethanol (g/L)	0.57 ± 0.32	0.56 ± 0.05	0.15 ± 0.06	ND	ND	0.92 ± 0.22

ND = not detected.

4. Discussion

The world production of olives relies on a few concentrated areas, namely, the Mediterranean Sea Basin, Australia, China, and some countries in North (USA and Mexico) and South (Peru, Argentina, and Chile) America. For instance, it has been reported that 290,000 tons of olives were processed in 2017 in Jordan, producing 175,000 m^3 of OMWWs [30]. Therefore, considering the production of 3.12 million tons of olives in 2019/2020 as provided by Statista, 2.6 million m^3 of OMWWs are potentially being generated. The magnitude of the problem is exacerbated by the absence of (efficient) wastewater treatment processes in place [30]. Therefore, processes that can contribute to COD removal are urgently needed. Filamentous fungi are able to assimilate a wide range of compounds, both organic and inorganic, and after recovery of the mycelium, can highly contribute for COD removal with concomitant production of value-added products. Wastewater characteristics including mass concentration of inhibitory compounds, lack of nutrients, content of suspended solids, and pH can influence fungal growth and composition of the produced fungal biomass. Therefore, these aspects were investigated in this work.

In the first stage of the study, the ascomycete *A. oryzae* was found to be a more robust microorganism, in comparison to the ascomycete *N. intermedia* and the zygomycete *R. delemar*, for growth in OMWW, achieving 8.1 g/L of biomass within 96 h cultivation. In an another study investigating the production of lipase enzyme, it was reported that biomass weight of two different *A. oryzae* strains were 5.4–5.7 g/L after 168 h in OMWW containing a comparatively lower COD of 25.6–51.5 g/L [12]. The motivation behind the use of these three strains is related to the different characteristics they can provide to processes. For instance, *N. intermedia* is a superior ethanol producer than *A. oryzae* [31], while the cell wall

composition of *R. delemar*, containing both chitin and chitosan, differs from that of the two ascomycetes, containing mostly chitin and no chitosan [32]. Although other cultivation parameters might have influenced the final output, several research studies have shown the superior performance of *A. oryzae* when its comparison with other strains, e.g., *N. intermedia*, was carried out. Examples include research works studying the growth on filamentous fungi in fish processing-derived wastewaters [19,20], vinasse [33], starch plant wastewater [34], and ethanol production-derived side streams [31].

Simple processing and extraction of value-added products can increase the feasibility of new bio-based processes with positive environmental impacts. Accordingly, the need of pH adjustment with addition of chemicals or of wastewater dilution increase the complexity of the process. The results from this study support the growth of *A. oryzae* directly in OMWW, where higher or comparable growth performance were obtained at initial pH of 5.2 in comparison to pH 6.5, and at varying mass concentrations of OMWW, respectively. *A. oryzae* lipase, produced during cultivation in fat-rich dairy products, was found to be significantly more active on fat degradation at pH >5.3 [16]; neutral pH conditions improved the enzymatic activity of *Aspergillus* species [35,36]; and a pH value of 6.5 was found to be suitable for growth of the ascomycete in vinasse [33]. However, adjusting the initial pH (6.1) of fish processing-derived wastewater to 5.0 led to a lower fungal growth performance [20]. Clearly, medium composition and physicochemical conditions play a role on fungal growth performance, and a tailored process development is needed while using wastewater of different origins [37]. OMWW can be toxic to microorganisms due to the presence of phenols and heavy metals. Inhibition of fungal growth has been reported in previous studies [37–39], and therefore OMWW or its phenols-rich extracts have been investigated as an antimicrobial agent. The growth of *Aspergillus* strains in undiluted OMWW, with or without nutrients addition has been reported for COD range of 25.6–123.7 g/L [12,40,41] pointing out the robustness of the genus for bioconversion of this wastewater.

Another factor during process development is productivity and, in this work, the cultivation time was reduced by 50% through addition of a nitrogen source. Similarly to the pH value for cultivation, the source of nitrogen should be tailored to the wastewater to go through bioconversion and, in the case of OMWW, it should also be tailored to the strain type and desired value-added product. For instance, NH_4Cl was found to be a more suitable nitrogen source than $(NH_4)_2SO_4$, $NaNO_3$, and urea for lipase production [41]. In addition to the need for finding the right nitrogen source, the results obtained point out the need for mixing different low-value substrates in order to attain satisfactory fungal growth and offset the need of addition of chemicals. Such strategy is commonly used in anaerobic digestion; however, it has not been so common in the research with filamentous fungi under submerged cultivation [14]. Co-cultivation strategies have, however, been applied in solid-state fermentation [23,42]. The first proposal for co-cultivation approaches is the addition of fish processing-derived wastewaters higher in protein and lower in carbohydrates [19,20]. The addition of yeast extract has also been reported to be needed to ensure fungal growth in OMWW [43], or leading to higher lipase production [41]. In order to enhance the feasibility of the process, it is proposed that yeast extract can be replaced by a filamentous fungal autolysate, obtained from fungal growth in low-value streams, as previously investigated [44]. Although in this study, the focus was on the effect of nitrogen addition, other types of nutrient unbalances might exist in OMWW and should be investigated in future studies.

Cultivation of *A. oryzae* in undiluted OMWW led to a protein content of w = 15.9%, which is in accordance with previous studies on growth of filamentous fungi in OWMM using both submerged and solid-state fermentation strategies. For instance, Hamdi et al. [21] have obtained a protein content range of w = 11.5–23% with *Aspergillus niger*, depending on OMWW composition and submerged cultivation time; Laconi et al. [22] have reported fungal biomass containing w = 13% protein following submerged growth of a fungal mixture in OWMM that had undergone an alkaline-oxidative treatment; and Giannoutsou et al. [23] have reported a product containing w = 19% protein following solid-state fermentation of *Paecilomyces variotii* on dried OMWW and molasses. Nonetheless, higher protein contents—within w = 42–58%—have been obtained following growth of, e.g., *Aspergillus oryzae* in

various low-value substrates such as vinasse [33] and thin stillage [31,45]. Therefore, even after addition of nitrogen and significant improvement of the protein content of the fungal biomass, a plethora of research studies have reported fungal biomass containing higher protein contents. Several research works have pointed out that the macroscopic filamentous structure and consequent entanglement with suspended solids might be related to lower protein contents. Examples include the growth of filamentous fungi in fish processing-derived sidestreams [19,20], thin stillage [46], and potato starch wastewater [34]. This study was pioneer on scrutinizing the effect of the content of suspended solids on the final protein content of the fungal biomass, where the highest protein content of w = 44.9% was obtained following growth in the supernatant, being similar to that reported in the literature and the highest reported, to the best of our knowledge, for microbial biomass obtained from growth in OMWW.

The COD reduction, following fungal cultivation and biomass separation, was 35–44%. Such COD reduction range is comparable to those reported in the literature using OMWW. For instance, 50% COD reduction was obtained under repeated batch process of *Pleurotus ostreatus* in OMWW (94.8 g/L initial COD) [40]; 78% removal was obtained after acclimatization of filamentous fungi in OMWW (82 g/L initial COD) [47]; and 55% removal after 74 h of cultivation of *Aspergillus niger* in bubble column (123.7 g/L initial COD) [48]. These comparisons might point out that there is room for improvement on COD removal by *A. oryzae*. The wastewater remaining after fungal cultivation and biomass separation still contains high chemical oxygen demand entailing further processing such as through activated sludge process. Therefore, the effect of a fungal cultivation on downstream processing also needs to be investigated in future studies.

Altogether, based on the results obtained in this study, it is proposed that research interest on bioconversion of OMWW into feed protein and COD removal should be renewed. Through close control of cultivation medium composition, high protein-containing fungal biomass can be obtained applying short and simple bioprocessing. Further research works should, e.g., investigate alternative nitrogen sources, preferably those that remain as waste, and their impact on protein content and COD removal, or cultivate filamentous fungi in bioreactor to take advantage of continuous supply of air. The integration of cultivation of filamentous fungi with membrane bioreactors should also be investigated. Similarly to filamentous fungi, membrane bioreactors function as selective sieves and can attain separation of several value-added streams, such as phenols-rich stream, and high-cell mass concentrations conditions. Such a strategy has widely been investigated for detoxification of industrial wastewaters containing a wide range of micropollutants [14].

5. Conclusions

A. oryzae is a robust microorganism for conversion of OMWW into protein-rich fungal biomass with concomitant COD removal. The significant increase in the protein content of fungal biomass through simple nitrogen addition and OMWW phase-separation might represent a basis for renewed research interest in the use of filamentous fungi for management of OMWW. It is hypothesized that laying efforts in the development of bioprocesses for the management of OMWW can benefit from the clustered availability of OMWW in the world.

Author Contributions: Conceptualization, M.J.T. and J.A.F.; methodology, T.S., M.O., M.J.T., and J.A.F.; formal analysis, T.S. and M.O.; investigation, T.S. and M.O.; writing—original draft preparation, T.S.; writing—review and editing, M.J.T. and J.A.F.; supervision, M.J.T. and J.A.F.; funding acquisition, M.J.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Swedish Agency for Economic and Regional Growth through a European Regional Development Fund.

Acknowledgments: We would like to thank Dizem Olive Oil Factory (Çanakkale, Turkey) for providing the olive oil mill wastewater.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Dermeche, S.; Nadour, M.; Larroche, C.; Moulti-Mati, F.; Michaud, P. Olive mill wastes: Biochemical characterizations and valorization strategies. *Process. Biochem.* **2013**, *48*, 1532–1552. [CrossRef]
2. Rinaldi, M.; Rana, G.; Introna, M. Olive-mill wastewater spreading in southern Italy: Effects on a durum wheat crop. *Field Crops Res.* **2003**, *84*, 319–326. [CrossRef]
3. Hentati, O.; Oliveira, V.; Sena, C.; Bouji, M.S.M.; Wali, A.; Ksibi, M. Soil contamination with olive mill wastes negatively affects microbial communities, invertebrates and plants. *Ecotoxicology* **2016**, *25*, 1500–1513. [CrossRef]
4. Niaounakis, M.; Halvadakis, C.P. *Olive-Mill Waste Management*; Typothito, George Dardanos: Athens, Greece, 2004.
5. Morillo, J.A.; Antizar-Ladislao, B.; Monteoliva-Sánchez, M.; Ramos-Cormenzana, A.; Russell, N.J. Bioremediation and biovalorisation of olive-mill wastes. *Appl. Microbiol. Biotechnol.* **2009**, *82*, 25–39. [CrossRef]
6. Silvan, J.M.; Pinto-Bustillos, M.A.; Vásquez-Ponce, P.; Prodanov, M.; Martinez-Rodriguez, A.J. Olive mill wastewater as a potential source of antibacterial and anti-inflammatory compounds against the food-borne pathogen Campylobacter. *Innov. Food Sci. Emerg. Techn.* **2019**, *51*, 177–185. [CrossRef]
7. Belaqziz, M.; Tan, S.P.; El-Abbassi, A.; Kiai, H.; Hafidi, A.; O'Donovan, O.; McLoughlin, P. Assessment of the antioxidant and antibacterial activities of different olive processing wastewaters. *PLoS ONE* **2017**, *12*, e0182622. [CrossRef]
8. Papazi, A.; Pappas, I.; Kotzabasis, K. Combinational system for biodegradation of olive oil mill wastewater phenolics and high yield of bio-hydrogen production. *J. Biotechnol.* **2019**, *306*, 47–53. [CrossRef] [PubMed]
9. Kıpçak, E.; Akgün, M. Biofuel production from olive mill wastewater through its Ni/Al2O3 and Ru/Al2O3 catalyzed supercritical water gasification. *Renew. Energy* **2018**, *124*, 155–164. [CrossRef]
10. Elisashvili, V.; Kachlishvili, E.; Asatiani, M.D. Efficient production of lignin-modifying enzymes and phenolics removal in submerged fermentation of olive mill by-products by white-rot basidiomycetes. *Int. Biodeterior. Biodegrad.* **2018**, *134*, 39–47. [CrossRef]
11. Abrunhosa, L.; Oliveira, F.; Dantas, D.; Gonçalves, C.; Belo, I. Lipase production by *Aspergillus ibericus* using olive mill wastewater. *Bioprocess. Biosyst. Eng.* **2013**, *36*, 285–291. [CrossRef] [PubMed]
12. D'Annibale, A.; Sermanni, G.G.; Federici, F.; Petruccioli, M. Olive-mill wastewaters: A promising substrate for microbial lipase production. *Bioresour. Technol.* **2006**, *97*, 1828–1833. [CrossRef] [PubMed]
13. Arous, F.; Azabou, S.; Jaouani, A.; Zouari-Mechichi, H.; Nasri, M.; Mechichi, T. Biosynthesis of single-cell biomass from olive mill wastewater by newly isolated yeasts. *Environ. Sci.Pollut. Res.* **2016**, *23*, 6783–6792. [CrossRef] [PubMed]
14. Ferreira, J.A.; VarjaniTaherzadeh, M.J. A critical review on the ubiquitous role of filamentous fungi in pollution mitigation. *Curr. Pollut. Rep.* **2020**. [CrossRef]
15. Karimi, S.; Mahboobi Soofiani, N.; Mahboubi, A.; Taherzadeh, M.J. Use of organic wastes and industrial by-products to produce filamentous fungi with potential as aqua-feed ingredients. *Sustainability* **2018**, *10*, 3296. [CrossRef]
16. Mahboubi, A.; Ferreira, J.A.; Taherzadeh, M.J.; Lennartsson, P.R. Production of fungal biomass for feed, fatty Acids, and glycerol by *Aspergillus oryzae* from fat-rich dairy substrates. *Fermentation* **2017**, *3*, 48. [CrossRef]
17. Mahboubi, A.; Ferreira, J.A.; Taherzadeh, M.J.; Lennartsson, P.R. Value-added products from dairy waste using edible fungi. *Waste Manag.* **2017**, *59*, 518–525. [CrossRef]
18. Thunuguntla, R.; Mahboubi, A.; Ferreira, J.A.; Taherzadeh, M.J. Integration of membrane bioreactors with edible filamentous fungi for valorization of expired milk. *Sustainability* **2018**, *10*, 1940. [CrossRef]
19. Sar, T.; Ferreira, J.A.; Taherzadeh, M.J. Bioprocessing strategies to increase the protein fraction of Rhizopus oryzae biomass using fish industry sidestreams. *Waste Manag.* **2020**, *113*, 261–269. [CrossRef]
20. Sar, T.; Ferreira, J.A.; Taherzadeh, M.J. Conversion of fish processing side-streams into feed ingredients through submerged cultivation of edible *Aspergillus oryzae*. *Syst. Microbiol. Biomanuf.* **2020**. [CrossRef]
21. Hamdi, M.; Khadir, A.; Garcia, J.-L. The use of *Aspergillus niger* for the bioconversion of olive mill waste-waters. *Appl. Microbiol. Biotechnol.* **1991**, *34*, 828–831. [CrossRef]
22. Laconi, S.; Molle, G.; Cabiddu, A.; Pompei, R. Bioremediation of olive oil mill wastewater and production of microbial biomass. *Biodegradation* **2007**, *18*, 559–566. [CrossRef] [PubMed]

23. Giannoutsou, E.P.; Katsifas, E.A.; Geli, A.; Karagouni, A.D. Protein increase and lysine production by a *Paecilomyces variotii* strain grown on two-phase olive mill waste. *World J. Microbiol. Biotechnol.* **2012**, *28*, 849–856. [CrossRef] [PubMed]
24. Adams, R.C.; MacLean, F.S.; Dixon, J.K.; Bennett, F.M.; Martin, G.I.; Lough, R.C. The utilization of organic wastes in N. Z. *N. Z. Eng.* **1951**, *6*, 396–424.
25. Sluiter, A.; Hames, B.; Hyman, D.; Payne, C.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D.; Wolfe, J. *Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples*; Technical Report No. NREL/TP-510-42621; National Renewable Energy Laboratory: Golden, CO, USA, 2008.
26. Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. *Determination of Ash in Biomass*; Technical Report No. NREL/TP-510-42621; National Renewable Energy Laboratory: Golden, CO, USA, 2008.
27. Ferreira, J.A.; Lennartsson, P.R.; Taherzadeh, M.J. Production of Ethanol and Biomass from Thin Stillage Using Food-Grade Zygomycetes and Ascomycetes Filamentous Fungi. *Energies* **2014**, *7*, 3872–3885. [CrossRef]
28. Mæhre, H.K.; Dalheim, L.; Edvinsen, G.K.; Elvevoll, E.O.; Jensen, I.-J. Protein determination—Method matters. *Foods* **2018**, *7*, 5. [CrossRef]
29. Zamani, A.; Jeihanipour, A.; Edebo, L.; Niklasson, C.; Taherzadeh, M.J. Determination of glucosamine and N-acetyl glucosamine in fungal cell walls. *J. Agric.Food Chem.* **2008**, *56*, 8314–8318. [CrossRef]
30. Khdair, A.I.; Abu-Rumman, G.; Khdair, S.I. Pollution estimation from olive mills wastewater in Jordan. *Heliyon* **2019**, *5*, e02386. [CrossRef]
31. Bátori, V.; Ferreira, J.A.; Mohammad, M.J.; Lennartsson, P.R. Ethanol and protein from ethanol plant by-products using edible fungi *Neurospora intermedia* and *Aspergillus oryzae*. *BioMed Res. Int.* **2015**. [CrossRef]
32. Ruiz-Herrera, J. Biosynthesis of the fungal cell wall. In *Pathogenic Fungi: Structural Biology and Taxonomy*; San-Blas, G., Calderone, R.A., Eds.; Caister Academic Press: Norfolk, UK, 2004; pp. 41–87.
33. Karimi, S.; Mahboobi Soofiani, N.; Lundh, T.; Mahboubi, A.; Kiessling, A.; Taherzadeh, M.J. Evaluation of filamentous fungal biomass cultivated on vinasse as an alternative nutrient source of fish feed: Protein, lipid, and mineral composition. *Fermentation* **2019**, *5*, 99. [CrossRef]
34. Souza Filho, P.F.; Zamani, A.; Taherzadeh, M.J. Edible protein production by filamentous fungi using starch plant wastewater. *Waste Biomass Valoriz.* **2019**, *10*, 2487–2496. [CrossRef]
35. Toida, J.; Kondoh, K.; Fukuzawa, M.; Ohnishi, K.; Sekoguchi, J. Purification and characterization of a lipase from *Aspergillus oryzae*. *Biosci. Biotechnol. Biochem.* **1995**, *59*, 1199–1203. [CrossRef] [PubMed]
36. Colla, L.M.; Ficanha, A.M.M.; Rizzardi, J.; Bertolin, T.E.; Reinehr, C.O.; Costa, J.A.V. Production and characterization of lipases by two new isolates of *Aspergillus* through solid-state and submerged fermentation. *BioMed Res. Int.* **2015**, *2015*, 725959. [CrossRef] [PubMed]
37. Vagelas, I.; Gougoulias, N.; Giurgulescu, L.; Papageorgiou, I. Potential antifungal activity of olive mill wastewater against postharvest diseases of pears. *Carpathian J. Food Sci. Technol.* **2012**, *4*, 31–36.
38. Bevilacqua, A.; Cibelli, F.; Raimondo, M.L.; Carlucci, A.; Lops, F.; Sinigaglia, M.; Corbo, M.R. Fungal bioremediation of olive mill wastewater: Using a multi-step approach to model inhibition or stimulation. *J. Sci. Food Agric.* **2017**, *97*, 461–468. [CrossRef]
39. Quaglia, M.; Moretti, C.; Cerri, M.; Linoci, G.; Cappelletti, G.; Urbani, S.; Taticchi, A. Effect of extracts of wastewater from olive milling in postharvest treatments of pomegranate fruit decay caused by *Penicillium adametzioides*. *Postharv. Biol. Technol.* **2016**, *118*, 26–34. [CrossRef]
40. Pesciaroli, L.; Petruccioli, M.; Federici, F.; D'Annibale, A. *Pleurotus ostreatus* biofilms exhibit higher tolerance to toxicants than free-floating counterparts. *Biofouling* **2013**, *29*, 1043–1055. [CrossRef] [PubMed]
41. D'Annibale, A.; Brozzoli, V.; Crognale, S.; Gallo, A.M.; Federici, F.; Petruccioli, M. Optimisation by response surface methodology of fungal lipase production on olive mill wastewater. *J. Chem. Technol. Biotechnol.* **2006**, *81*, 1586–1593.
42. Gmoser, R.; Fristedt, R.; Larsson, K.; Undeland, I.; Taherzadeh, M.J.; Lennartsson, P.R. From stale bread and brewers spent grain to a new food source using edible filamentous fungi. *Bioengineered* **2020**, *11*, 582–598. [CrossRef]
43. Jallouli, R.; Bezzine, S. Lipase production by a Tunisian *Fusarium solani* strain cultivated on olive oil wastewater-based media and a biotreatment assay. *Desalin.Water Treat.* **2016**, *57*, 20327–20331. [CrossRef]
44. Asachi, R.; Karimi, K.; Taherzadeh, M.J. Fungal autolysate as a nutrient supplement for ethanol and chitosan production by *Mucor indicus*. *Biotechnol. Lett.* **2011**, *33*, 2405–2409. [CrossRef]

45. Ferreira, J.A.; Lennartsson, P.R.; Taherzadeh, M.J. Production of ethanol and biomass from thin stillage by *Neurospora intermedia*: A pilot study for process diversification. *Eng. Life Sci.* **2015**, *15*, 751–759. [CrossRef]
46. Rasmussen, M.L.; Khanal, S.K.; Pometto, A.L.; van Leeuwen, J. Water reclamation and value-added animal feed from corn-ethanol stillage by fungal processing. *Bioresour. Technol.* **2014**, *151*, 284–290. [CrossRef] [PubMed]
47. Aissam, H.; Penninckx, M.J.; Benlemlih, M. Reduction of phenolics content and COD in olive oil mill wastewaters by indigenous yeasts and fungi. *World J. Microbiol. Biotechnol.* **2007**, *23*, 1203–1208. [CrossRef]
48. Hamdi, M.; Ellouz, R. Bubble column fermentation of olive mill wastewaters by *Aspergillus niger*. *J. Chem. Technol. Biotechnol.* **1992**, *54*, 331–335. [CrossRef]