

**GROWTH OF FILAMENTOUS FUNGI IN PURE
OLIVE OIL: A FUNDAMENTAL STUDY FOR
APPLICATION TO VEGETABLE OIL-DERIVED
WASTE STREAMS**

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Abstract

Vegetable oil is more difficult to degrade by microorganisms in comparison to carbohydrates and protein. Thus, it creates serious environmental and health concerns if oil-derived waste streams produced by restaurants and industries remain untreated. In this study, a strategy has been developed to grow filamentous fungi in pure olive oil so that it can be used as a benchmark for growth in olive oil mill sidestreams. The growth of different strains (*Aspergillus oryzae*, *Neurospora intermedia* and *Rhizopus oryzae*) was tested in pure olive oil. A pre-germination stage using glucose as carbon and energy source, or the addition of yeast extract, were found necessary for successful fungal growth in olive oil. Here, *A. oryzae* showed a superior performance in comparison to *N. intermedia* and *R. oryzae*. Medium pH did not impact *A. oryzae* growth in olive oil, whereas a concentration higher than 40 g/L of the latter impaired the growth of the ascomycete. Obtained biomasses from *A. oryzae* and *N. intermedia* cultivations in olive oil were analyzed and compared for protein, fat, ash, and alkali-insoluble material (cell wall content), where the presence of olive oil had a steering effect. The fungal biomass of *A. oryzae*, obtained from cultivation in the absence of olive oil, contained 0.33% fat and 48% protein, whereas the respective values in the presence of olive oil were 31% and 14%. Similar trends on fat and protein contents were observed for the biomass of *N. intermedia*. Sudan black staining was also performed on fresh biomass which clearly indicated the presence of oil globules inside the fungal cells. This research can be a fundamental step towards treatment of oil-based waste streams, which entails high-energy and costs if treated, or environmental impacts during informal discharges. Moreover, the fact that the composition of fungal biomass can be steered through addition of olive oil increases the versatility of the originated biomass for various applications, namely in feed, food and biofuel production.

Keywords: filamentous fungi, oleaginous fungi, *Aspergillus oryzae*, *Neurospora intermedia*, olive oil, fat, fungal biomass

1. Introduction

The advancement in agriculture and food technology, with many valuable products, produces concomitantly large amounts of waste. This waste is generally organic rich and it can thus cause serious environmental problems, if discarded into the environment without suffering efficient wastewater treatment (Arvanitoyannis and Ladas, 2008). Among other wastes, fat and oil are the major by-products of many industries such as agricultural and tanning industries, and slaughterhouses. These industries are facing a major problem to dispose of their organic waste which can cause serious environmental concerns if not utilized and remained untreated. For instance, the growth of pathogenic microorganisms in the fat-rich waste can easily happen due to its high organic content, which, in turn, can cause the spread of pathogenic diseases and foul smelling by the degradation of fatty acids. It also produces many greenhouse gases if landfilled (Jayathilakan et al., 2012).

Olive oil mill wastewater is originated during the production of olive oil, and contains high amounts of phenolics and tannins. In addition to the large amount of oil and protein, phenolics and tannins can have an inhibitory effect on microbial growth. Thus, domestic wastewater treatment method is not being used for the treatment of olive mill wastewater. In most of the cases olive oil mill wastewater is being directly discharged into the sewer systems or left in the open fields to dry up the solid contents which chemically produce large amount of toxins and foul smelling in the environment (Nieto et al., 2011).

Filamentous fungi can grow on a wide range of substrates as a result of the diversified enzymatic machinery including the production of e.g cellulase, xylanase, protease and lipase. Therefore, they are of potential for valorization of fat-rich substrates given their ability to produce lipases to convert fat into short-chain fatty acids and esters with applications in several industrial sectors (Andualema and Gessesse, 2012). Particularly, the use of edible filamentous fungi, widely used in the manufacturing of human fermented food such as red kojic rice, tempe, soy sauce, rice wine, Chinese liquor and tofu, can open up the possibility of production of fungal-based products for feed and food applications. Furthermore, in addition to the nutritious fungal biomass, these filamentous fungi can be used for the production of organic acids and ethanol (Ferreira, 2015).

The overall aim of this research study was to develop fungal-based management strategies for oil rich sidestreams of olive oil mill wastewater. We studied the behavior of edible filamentous

fungi (zygomycetes and ascomycetes) when grown in supermarket-available olive oil to be used as a bench-mark for growth in olive oil mill sidestreams.

2. Background

2.1. Fat and oil waste management

Fat is a byproduct of many industries such as slaughterhouses, restaurants, and dairy and tanning industries. Consequently, their wastewater together with fat-containing wastewater from households can lead to blockage of sewage system by the formation of fat, oil and grease known as FOG (Wallace et al., 2017).

Table 1: Lipid profile of olive oil, butter, whey, pork and cow fat.

Fatty Acid	Class	Olive Oil	Butter	Whey	Pork fat	Cow fat
Myristic Acid	C14:0	0	11.1	9.7	1.0	2.8
Palmitic Acid	C16:0	16.62	33.7	31.0	21.1	26.8
Palmitoleic Acid	C16:1	1.86	1.0	1.1	1.5	2.05
Stearic Acid	C18:0	2.70	10.4	15.4	11.5	34.9
Oleic Acid	C18:1	61.28	21.78	26.4	40.1	30.2
Linoleic Acid	C18:2	16.47	-	6.6	21.7	0.7
Linolenic Acid	C18:3	0.61	-	-	1.5	-
Other	-	0.46	15.4	9.8	2.3	-
Source	-	(Mattos et al., 2004)	(Gun and Simsek, 2011)	(Boyd et al., 1999)	(Sarantopoulos et al., 2014)	(Selvam and Vadivel, 2012)

Olive oil, the major oil consumed worldwide, is mainly composed of unsaturated fatty acids (94%) in comparison to other fats e.g. butter, whey, pork and cow fat (Table1). Olive oil industry originates a lot of waste during the production of olive oil, called olive mill wastewater. The olive mill wastewater contains large amount of protein, lipids, poly-alcohols as well as

small amount of oil (up to 10%), depending upon the production process used. Many methods are being used to treat olive mill wastewater such as evaporation of water in open fields which also produces large amount of toxins and foul smelling. Microbial treatment can be used to produce valuable compounds such enzymes (e.g. lipase) and biomass, for feed applications, from organic rich olive mill wastewater (Crognale et al., 2006, Brozzoli et al., 2009a).

The main products in the dairy industry are cheese and butter. These wastes possess different amounts of fat. Cheese manufacturing produces large amounts of whey as a byproduct. Nowadays, cheese whey, containing 0.4% fat, is used as e.g. a nutrient source for microbial growth or supplement for body-building. A byproduct of the butter manufacturing process is butter milk, containing 0.9% fat, which is normally used as animal feed (Ryan and Walsh, 2012).

Fat is also a major byproduct of the meat industry. The percentage of fat present in cow and pork is 4 and 3%, respectively. The fat removed from the clean tissues of cow and pork is consumed as a food named tallow and lard (Jayathilakan et al., 2012). Animal fat is also used in the manufacturing of soap, lubricant, insecticide, biodiesel and feed, etc. (Bondioli et al., 2019, Leoci, 2014). Animal fat contains saturated and monounsaturated fatty acid contents, which account to around 88%, while polyunsaturated fatty acid account to about 12% (Bondioli et al., 2019). Fatty acid composition of different sources of waste pork and cow fat is presented in **Table 1**.

2.2. Chemical and mechanical waste treatment

Fat and oil wastes can be converted into biodiesel (used as a biofuel) and glycerol (used as a biopolymer) by a transesterification process (Wallace et al., 2017). Other methods are used to dispose of fat-containing waste, such as rendering, in which the waste material from the meat industry is mechanically treated and separated into different products that can be used as a low-cost raw material for production of other products (Franke-Whittle and Insam, 2013). Alkaline hydrolysis, in which the material is degraded into smaller molecules by the action of sodium and potassium hydroxide (Kalambura et al., 2011) is another waste management and valorization alternative.

2.3. Biological waste treatment

The biological waste treatment method of fat has many advantages over non-biological methods e.g. low cost (Pham et al., 2015) and the possibility to use a wide range of species during the

treatment, leading consequently to the production of diverse value-added products such as enzymes and biomass. The latter can be used as a raw material for many other industries (Crini and Lichtfouse, 2019). The use of bacteria for the anaerobic treatment of organic waste produces a large amount of sludge which requires further treatment before it can be used as a fertilizer. On the contrary, filamentous fungi produce organic acids, enzymes and a large amount of biomass under aerobic conditions, which can be used for food or feed purposes (Sankaran et al., 2010), leaving potentially behind a stream low in organic matter and easy to purify. Zygomycetes and ascomycetes are two groups of filamentous fungi with the capacity to grow on variable substrates and produce a wide range of products such as enzymes, organic acids and biomass (Ferreira et al., 2016).

2.4. Fungal biorefineries

Zygomycetes and ascomycetes have widely been investigated for the establishment of biorefineries consuming different sources of carbohydrates, fats and proteins i.e., able to consume a large number of substrates from monomers to polysaccharides including starch and lignocellulose (Kumar and Ray, 2014). Submerged fermentation (SmF) and solid-state fermentation (SSF) are the two most common techniques used for the fungal cultivation. SmF is dominant at both experimental and applied levels related to superior substrate utilization and heat transfer (Ferreira et al., 2016).

Many enzymes have been produced through fungal cultivation including cellulases for the degradation of lignocellulosic biomass and lipases for the degradation of lipopolymers. Moreover, many ascomycetes strains have been used for production of ethanol, citric acid (widely used in the food industry), gluconic acid (used in many applications such as medicine, textile and food), and itaconic acid (used in the polymer industry). Filamentous fungi have also been widely used in the food industry, for production of various human fermented foods. Examples include tempe produced by *Rhizopus oryzae*, oncom by *Neurospora intermedia*, miso by *Aspergillus oryzae* and red rice by *Monascus purpureus*. Given the relevant levels of protein, fat, essential amino acids and polyunsaturated fatty acids, the fungal biomass obtained through cultivation of edible fungal strains has also been considered for feed applications such as for poultry, fish and cattle (Ferreira et al., 2016).

2.5. Fat and oil as fungal substrates

Microbial lipases have many applications such as in the production of biofuel, cosmetic, food, medicine, organic acids, and esters. Lipases have been produced by many fungal species e.g. *Candida rugosa*, *Candida antarctica*, *Thermomyces lanuginosus*, *Rhizomucor miehei*, *Pseudomonas*, *Mucor*, *Geotrichu*, *Rhizopus rhizopodiformis* A13, *Rhizomucor pusillus* A16, *Aspergillus niger* MTCC2594, *Penicillium restrictum*, *Rhizopus oligosporous*, *Aspergillus niger* NCIM 1207, *Rhizopus delemar* and *Aspergillus oryzae*. A wide range of substrates can be utilized by fungi to produce lipases e.g. rice bran, wheat bran, gingelly oil cake, almond meal, mustard oil cake, neem oil cake, groundnut oil cake, gingerly seed and groundnut kernel. These substrates are used routinely for lipase production by SSF (Kumar and Ray, 2014).

Mahboubi et al. (2017) have also reported the successful use of dairy waste as a fungal growth nutrient medium, despite the high existing lactic acid concentrations. Dewi et al. (2019) have isolated a *Aspergillus* sp. from batik wastewater wells, which through cultivation on the fat substrate available in batik wastewater, led to the concomitant reduction of heavy metals (e.g. Cr), phenol, ammonia and sulfide compounds. Three strains of fungi i.e. *Aspergillus niger*, *Geotrichum candidum* and *Mucor miehei* were shown to be able to grow on beef and poultry fat using SSF and SmF cultivation strategies. Biomass concentrations for three different strains of fungi namely *A. niger*, *G. candidum* and *M. miehei*, after cultivation in medium enriched with beef fat, were 2.9, 4.2 and 1.4 g/L in SSF cultivation, 2.8, 3.9 and 1.8 g/L using SmF in shake-flasks and 1.6, 1.4 and 1.4 g/L using bioreactors, respectively. The percentages of fat utilization on SSF cultivation by *A. niger*, *G. candidum* and *M. miehei* were 17, 51, and 61%, using shake-flasks were 22, 23 and 19%, and using bioreactors 33, 20, and 16%, respectively. Thus, maximum growth and fat utilization depend upon the cultivation condition and type of fungus used (Bednarski et al., 1993). The growth of an *Aspergillus* strain in SSF using agro-industrial waste, i.e. cottonseed cake, rice and red gram husk, has also been reported (Nema et al., 2019). In another study, lipase production from an *Aspergillus* strain as well as its temperature, pH stability and activity were characterized and compared under both SSF and SmF. The lipase obtained through SmF had an optimum temperature of 37 °C and pH 7.2, while the lipase obtained through SSF had an optimum temperature of 35 °C and pH 6.0. It was also shown that the lipase obtained through SmF was more stable than the lipase obtained through SSF (Colla et al., 2015). Lipases were also produced during the growth of three different strains of fungi namely *Penicillium chrysogenum*, *Trichoderma harzianum* and *Aspergillus flavus* on castor oil cake and olive oil in SSF. In this study, three factors were monitored namely pH,

cultivation time and substrate type. The authors have found that the cultivation time had a greater effect on the production of lipase than the pH, while the type of substrate did not have a major effect on lipase production. Among the three strains studied, the amount of lipase produced by *A. flavus* was five to nine times more than that produced by *P. chrysogenum* and *T. harzianum* (Toscano et al., 2013). In another study, the fungal species *Conidiobolus nanodes*, *Entomophthora exitalis*, *Mortierella isabellina*, and *Mucor circinelloides*, when grown on different vegetable oils (sesame oil, sunflower oil, linseed oil and mortierella oil) led to biomass concentrations of 9 to 11 g/L, 5 to 8 g/L, 7 to 9 g/L, 4 to 7 g/L and fat contents of 26% to 44%, 25% to 48%, 20% to 46% and 30% to 42% w/w, respectively (Kendrick and Ratledge, 1996). The growth of the fungal species *Aureobasidium melanogenum* was also investigated using 1% olive oil and 1% linseed oil. Initially, the biomass was grown on 0.2% glucose and 0.2% oil followed by the addition of 1% vegetable oils (olive and linseed oil). The fungal growth was monitored by measuring the cell counts, where 10^8 cells/ml were obtained in comparison to the initial concentration of 10^5 cells/ml. Nile red staining was also performed which confirmed the presence of fatty acids inside the cells (van Nieuwenhuijzen et al., 2019).

2.6. Social and ethical aspects:

Organic wastes such as food, wood, straw, sludge, manure etc. represent 70 percent of total waste around the world. These wastes are highly rich in organic compounds and a potential source of energy for microorganisms. If this highly organic rich waste remains untreated then pathogenic microorganisms deteriorate organic compounds into toxins and foul smelling (Zhao et al., 2017). This ultimately leads to environmental pollution with concomitant depletion of natural resources. In an increasingly industrialized world together with population increase forecasts, the need of pollution mitigation, while attaining food and energy security becomes crucial. One potential solution is develop circular bioeconomies where wastes materials are not discarded but converted into feed, food, energy and chemicals the society needs. This can contribute to waste management but also to the production of biofuels, biochemicals and biopolymers that can replace fossil fuels-based products in human activities with additional environmental benefits.

Filamentous fungi, which are eukaryotic saprotrophic microorganisms having unique capabilities to degrade toxic compounds in decaying waste organic matter with the help of their enzymatic machinery, can contribute to the reduction of organic waste as well as to the production of valuable products (Ferreira et al., 2016, Araceli and Hugo, 2019). Filamentous fungi are usually used in the form of food for decades. So, the use of edible filamentous fungi

in waste treatment applications is better than the use of bacteria in a way that it cannot cause any adverse effect to the environment and pathogenic effect in human or animals.

CO₂, CH₄, and NO_x are the major contributors of greenhouse gas emissions during landfilling, and aerobic and anaerobic waste degradation. Growth of edible filamentous fungi on waste streams can help decreasing greenhouse gas emissions as well as generating wealth for the stakeholders with the production of valuable products in terms of metabolites or biomass. Edible filamentous fungi can also provide a solution to the increased demand of food and feed products of better nutritional quality that could help enhance food safety and security globally (Chen et al., 2015, Sibirny, 2017, Vijayan et al., 2017). This thesis presents insights on the use of olive oil which can become the foundation for the valorization of sidestreams from olive oil mills. Therefore, the present thesis can give a contribution to waste management, while opening the window to potential alternative protein and fat sources for human consumption and feed purposes. The presented thesis can, therefore, have an environmental, social, and economic impact in the society.

3. Methodology

3.1. Culture preparation

Eight different filamentous fungal strains, belonging to the group of ascomycetes and zygomycetes, were used throughout this work. The ascomycetes strains used were *Aspergillus oryzae* var. *oryzae* CBS 819.72, isolated from Indonesian fermented food koji and obtained from Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands), *Fusarium venenatum* ATCC 20334 (American Type Culture Collection, Manassas, VA, USA) and *Neurospora intermedia* CBS 131.92, isolated from peanut based fermented food called oncom (Centraalbureau voor Schimmelcultures, The Netherlands). The zygomycetes strains were *Rhizopus oligosporus* ACM 145F (American Type Culture Collection, Manassas, VA, USA), *Rhizopus oligosporus* var. *microsporus* and *Rhizopus oryzae* CCUG 28958 collected from Culture Collection University of Gothenburg (Sweden) and *Rhizopus* sp. identified as RM4 CCUG 61147 collected from Culture Collection University of Gothenburg (Sweden). The fungal strains were maintained in Potato Dextrose Agar (PDA) medium containing 20 g/l glucose, 15 g/l agar and 4 g/l potato powder; the medium was autoclaved at 121 °C for 20 minutes. A pre-grown fungal plate was flooded with 20 ml sterile distilled water and a L-shape disposable plastic spreader was used to bring the spores into the solution; a volume of 0.1 ml

of the spore solution was then inoculated into new PDA plates. The inoculated plates were incubated at 30 °C for 3 days and stored at 4 °C until use.

3.2. Shake flask cultivation

3.2.1. Effect of medium recipe on fungal growth

Spore suspension was prepared from previous prepared plates by flooding with 20 ml milli-Q water. During all the experiments 250 ml cotton-plugged Erlenmeyer flasks were prepared with 100 ml of medium according to recipes presented in table 2.

Table 2. Media preparation for flask cultivation

Recipe	Fungal strain	Glucose (g/l)	Olive oil (g/l)	C/N	vitamin (ml/l)	MgSO ₄ .7H ₂ O (g/l)	KH ₂ PO ₄ (g/l)	CaCl ₂ .2H ₂ O (g/l)	Trace metal (ml/l)
1	<i>Ao, Ni, Fv, Ro,Rom, RM4, R. ory*</i>	0	28						
2	<i>Ao, Ni, Fv, Ro,Rom, RM4, R. ory*</i>	0	28	1	1				10
3	<i>Ao, Ni, Fv, Ro,Rom, RM4, R. ory*</i>	10	28	1	1				10
4	<i>Ao</i>	20	0	5	1	2.25	3.5	1	10
5	<i>Ao</i>	20	1	5	1	2.25	3.5	1	10
6	<i>Ao</i>	20	2.5	5	1	2.25	3.5	1	10
7	<i>Ao</i>	20	5	5	1	2.25	3.5	1	10
8	<i>Ao</i>	20	10	5	1	2.25	3.5	1	10
4	<i>Ao</i>	20	0	5	1	2.25	3.5	1	10
8	<i>Ao</i>	20	10	5	1	2.25	3.5	1	10
9	<i>Ao</i>	0	10	5	1	2.25	3.5	1	10
10	<i>Ao</i>	2	10	5	1	2.25	3.5	1	10
9	<i>Ao, Ni, Fv, Ro,Rom, RM4, R. ory*</i>	0	10	5	1	2.25	3.5	1	10
8	<i>Ascomycetes (Ao, Ni, Fv)</i>	20	10	5	1	2.25	3.5	1	10
8	<i>Zygomycetes (Ro, Rom, RM4, R. ory)</i>	20	10	5	1	2.25	3.5	1	10
8	<i>Zygomycetes (Ro, Rom, RM4, R. ory)</i>	20	10	5	1	2.25	3.5	1	10

All the flasks were prepared in duplicate and autoclaved at 121 °C for 20 minutes and inoculated with 2 ml spore suspension after pH adjustment to 5.5. Ten ml of spore suspension were stored for spore counting and 0.1 ml spore suspension was used for inoculation of new PDA plates. Spore concentration was measured by placing a sample in a Bürker's counting chamber. Spore concentrations were as follows: *Aspergillus oryzae* 3.9 x 10¹¹, *Fusarium venenatum* 5.1 x 10⁶, *Neurospora intermedia* 2.3 x 10⁸, *Rhizopus oligosporus* 4.9 x 10⁸, *Rhizopus oligosporus var. microsporus* 3.1 x 10⁹, *Rhizopus oryzae* 4.1x10⁸, and *Rhizopus sp. (RM4)* 1.1 x 10⁹ spores.mL⁻¹

¹. The flasks were incubated at 35 °C for 96 hours using a water bath shaking at 100 rpm. After 96 hours, pH and volume were measured. The biomass produced was harvested using a kitchen sieve (1 mm² pore size) and washed three times with milli-Q water. The biomass dry weight was measured according to Ferreira et al. (2014a) using the oven method by drying the biomass samples at 70 °C and freeze dried method until constant weight.

3.2.2. Effect of pH on fungal assimilation of oil

Effect of pH on fungal assimilation of oil was measured by preparing fungal media (table 2, recipe 8) with glucose as sole carbon source. After 32 hours of cultivation, the pH was adjusted at two different levels i.e. 5.8 and 6.5 together with the addition of 10 g/l oil and the cultivation continued until 100 h (ascomycetes) and 125 h (zygomycetes).

3.2.3. Effect of oil concentration

Effect of oil concentration on fungal biomass growth was investigated by using different oil concentrations. *A. oryzae* was first grown on glucose followed by the addition of different oil concentrations, namely 10, 20, 40, 60, 80 and 100 (g/L) after 32 hours of growth in glucose-containing medium. Samples were taken for HPLC over time and biomass was analyzed and compared after 100 hours of cultivation.

3.2.4. Effect of glycerol on *A. oryzae*

A. oryzae behavior on oil assimilation was measured by growing it solely on i) glycerol, ii) glucose and glycerol, iii) glucose and glycerol (addition after 32 hours) and iv). glucose and addition of glycerol (12 g/L) after 32 hours together with different concentrations of olive oil, namely 5, 10, 20, 40 and 80 g/L. Samples were taken for HPLC over time and biomass was analyzed and compared after 100 hours of cultivation.

3.2.5. Effect of yeast extract on biomass growth

Effect of yeast extract on biomass growth was measured using two different recipes described in table below.

Table 3. Media recipes of biomass growth in yeast extract

Recipe	Fungal strain	Yeast Extract (g/l)	Olive oil (g/l)	C/N	vitamin (ml/l)	MgSO ₄ .7H ₂ O (g/l)	KH ₂ PO ₄ (g/l)	CaCl ₂ .2H ₂ O (g/l)	Trace metal (ml/l)
1	<i>Ao</i>	5	10	5	1	2.25	3.5	1	10
2	<i>Ao</i>	5	10						

3.3. HPLC analysis

1.5 ml of sample was taken from each flask under aseptic conditions during cultivation and stored at -22 °C for further HPLC analysis of glucose, ethanol and glycerol concentration. Samples stored at -22 °C for HPLC analysis were thawed, vortexed and centrifuged for 10 min at 10,000 x g. 0.8 ml of supernatant was transferred to HPLC vials for analysis. Samples were analyzed by HPLC (Walters 2695, Walters Corporation, Milford, USA) using a hydrogen-based ion-exchange column (Aminex HPX-87H, Bio-Rad, Hercules, USA) at 60 °C with eluent of 5 mM H₂SO₄ at the rate of 0.6 mL/min. Each sample was put on analysis for 27 minutes.

3.4. Kjeldahl analysis

Total protein content in dry biomass was measured by analyzing nitrogen content using Kjeldahl block digestion and steam distillation. A InKjel P digester and a behrotest® S1 distiller 191 (behr Labor - Technik, Düsseldorf, Germany) were used for the analysis of the nitrogen content. The digestion was carried out in reaction vessels by using 20 ml of 98% (v/v) sulphuric acid, antifoam and KT1 tablets (Thompson & Capper ltd, Cheshire, United Kingdom). 0.5 g ± 0.05 g of dried biomass was used to analyze the nitrogen content in the digester for 100 minutes at 100% power. The digestate was then neutralized in the distillation unit by distillation for 5 minutes with 32% sodium hydroxide, followed by the collection of distillation vapors in the 50 ml of 4% boric acid solution. Acid base titration was then carried out using 0.1 N HCl until reaching the end point, namely of pH 4.5. The crude protein content was calculated by applying a nitrogen-to-protein conversion factor of 6.25.

3.5. Fat analysis

Fat contents in fungal biomass were analyzed using 0.2 g (±0.01) of freeze-dried biomass which was dissolved in 50 ml of water and centrifuged at 10,000×g for 5 minutes. The supernatant was taken and dissolved in 50 ml of organic solvent with 40:40:20 ratio of petroleum ether, diethyl ether and absolute ethanol. The content was mixed and phases were separated using a 250 ml separating funnel. The organic phase was collected in a 250 ml beaker and the aqueous phase was mixed again with 50 ml of organic solvent mixture, subjected to phase separation and collection in the same 250 ml beaker. The beaker containing the organic solvent mixture was left overnight in the fume hood at room temperature followed by drying at 105 °C for 1 hour. The amount of fat was measured by using the beaker weight difference (Majdejabbari et al., 2011).

3.6. Ash analysis

For ash analysis, 0.2 g of fungal biomass in crucible was ignited in a muffle furnace (Gallenkamp, London, UK) for 16 hours at $575\text{ }^{\circ}\text{C} \pm 25\text{ }^{\circ}\text{C}$ followed by 1 hour desiccation. The amount of ash was measured by using crucible weight difference (Sluiter et al., 2008).

3.7. Alkali insoluble material (AIM)

AIM was measured by dissolving 0.2 g (± 0.01) of biomass in 0.5 M NaOH (with the ratio of 30:1 v/w) and autoclaved at $121\text{ }^{\circ}\text{C}$ for 20 minutes. AIM was separated by centrifugation at 4,000 RPM and washed with distilled water until the neutral pH was obtained. AIM was dried at $70\text{ }^{\circ}\text{C}$ followed by one hour desiccation (Asadollahzadeh et al., 2018). The amount of AIM was measured by using crucible weight difference (Sluiter et al., 2008).

3.8 Sudan black staining

Sudan black staining was used for detection of intracellular lipid contents according to Burdon (1946) with little modification. Fresh fungal cells were heat fixed and flooded with 0.3% sudan black stain (prepared in 70% ethanol) with 10 minutes incubation. Excess stain was washed with 70% ethanol. Cells were counterstained with safranin for 10 seconds, washed with distilled water and examined under microscope at 1000x magnification.

3.9. Data analysis

All the experiments carried out in this research work were done in duplicate. Microsoft Excel was used for data analysis, namely the determination of average values and standard deviations. All error bars presented in the graphs represent 2 times the standard deviation.

4. Results

The main goal of this thesis work was to develop bioconversion strategies to attain successful assimilation of pure olive oil by edible filamentous fungi so that the same strategy can be utilized to oil rich waste streams derived from e.g. olive oil mills. Different medium recipes were used to grow ascomycetes and zygomycetes filamentous fungi into biomass in oil rich media. In addition to the medium recipe, the effect of fungal strain, pH, and oil concentration on fungal biomass growth was investigated. Comprehensive fungal biomass compositional analysis was also carried out in order to reveal the effect of addition of olive oil on e.g. fat and protein contents.

4.1. Effect of medium recipe on fungal growth

Effect of different growth media on fungal strains' growth was studied by supplementing the media with olive oil 28 g/L, olive oil 28 g/L + nitrogen source + trace metals + vitamins, and olive oil 28 g/l + nitrogen source + trace metals + vitamins + salts. Fully-grown biomass was not observed in any of the above mentioned recipes, irrespective of the fungal strain while using olive oil as sole carbon source in the medium. When the full medium was used spore germination into visible hyphae was observed; however, it failed to build a macroscopic and dense network. Olive oil has high amount of phenolic compounds that have been reported to inhibit bacterial and fungal growth (Diallinas et al., 2018). However, other factors might have hampered successful growth of filamentous fungi in the above medium recipes. The cultivation strategy was therefore modified to motivate fungal growth on olive oil. Different growth media for *Aspergillus oryzae* (*A. oryzae*) were prepared, supplemented with glucose (0, 2 and 20 g/L) and olive oil (0, 1, 2.5, 5 and 10 g/L) in addition to nitrogen, trace metals, vitamins and salts. Fungal growth was observed for *Aspergillus oryzae* in all recipes where glucose was present. Final pH was measured at the end of the experiment and the result is presented in Table 4. The initial pH that was set at 5.5 increased possibly due to assimilation of nitrogen source. Fungi require different growth components for its growth such as carbon and nitrogen sources, trace metals, salts, minerals and vitamins (van Nieuwenhuijzen et al., 2019). Supplementing the media with glucose and nutrients led to fungal biomass production that created the necessary conditions to attain further fungal growth upon addition of olive oil.

Table 4: Final pH obtained after *A. oryzae* cultivation in different medium recipes.

		Fungal strain	Glucose (g/l)	Olive oil (g/l)	Final pH (Initial pH 5.5)
Experiment 1	Recipe 4	AO	20	0	7.59
	Recipe 5	AO	20	1	7.98
	Recipe 6	AO	20	2.5	7.87
	Recipe 7	AO	20	5	7.85
	Recipe 8	AO	20	10	7.84
Experiment 2	Recipe 4	AO	20	0	7.73
	Recipe 8	AO	20	10	7.18
	Recipe 9	AO	0	10	5.36
	Recipe 10	AO	2	10	5.8

Different growth morphologies of *Aspergillus oryzae* were observed with the media containing oil vs no oil. The fungus grew as fluffy pellets around oil droplets in the presence of oil (Figure

1A) while small compact pellets were observed in the absence of oil in the medium (Figure 1B).

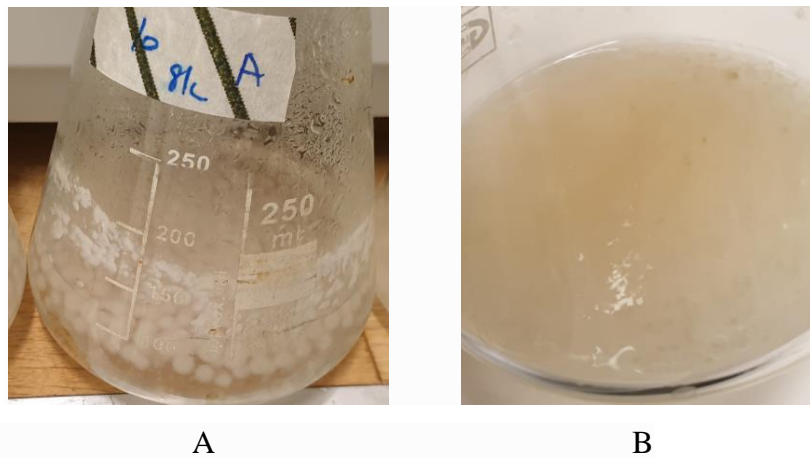


Figure 1. *A. oryzae* pellets with, A: olive oil 10 g/L and B: 20 g/L glucose.

As we can see in table 3 and figure 1 and 2, an increase in biomass concentration was observed concomitantly with an increase in the olive oil concentration with a yield % (g biomass/g substrate added) of 28, 33, 35, 34, 36, 14 and 80%. It was observed that using only oil as a carbon source (Table 3, Recipe 9), 1.37 g/L biomass was produced. This is possibly due to the fact that the fungus had very little access to oil probably due to oil and water phase separation. In order to enhance the contact of fungi with oil, the medium was manipulated with the addition of glucose that primarily helped fungus to germinate, resulting in the increased contact of fungal biomass with top layered oil. Up to 10.67 g/L biomass were produced in the presence of olive oil in comparison to 5.63 g/L biomass with only glucose as a carbon source, and 1.37 g/L biomass with only oil as a carbon source (Figure 2).

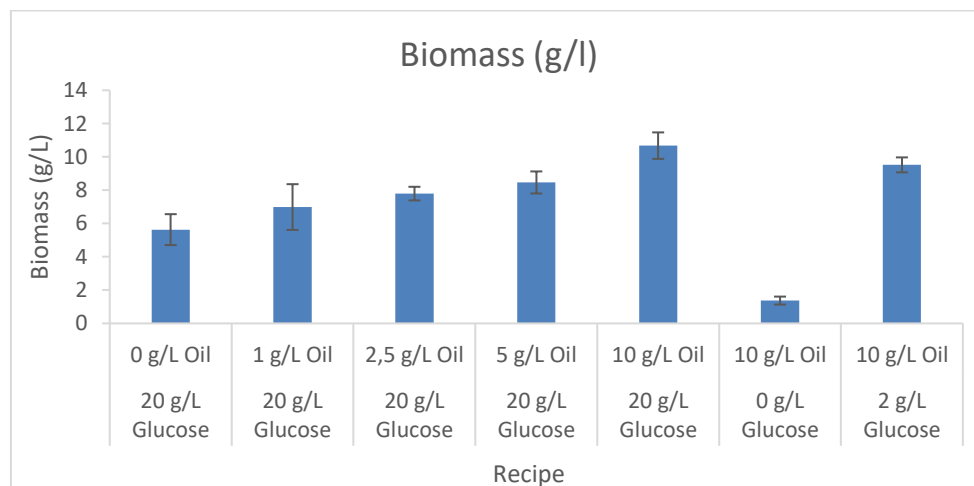


Figure 2: Dry biomass weight of *A. oryzae* after cultivation in the different medium recipes presented in Table 3.

For the identification of metabolites, high performance liquid chromatography (HPLC) was performed and the results are presented in Figure 3. In all samples, glucose was completely consumed after around 40 hours of incubation which points out absence of inhibition by the addition of olive oil after 32 hours. Ethanol produced by the metabolism of glucose was also consumed after around 80 hours of incubation. However, it was observed that, the fungus consumed oil as a carbon source and produced glycerol when other carbon sources become depleted in the medium (Figure 3).

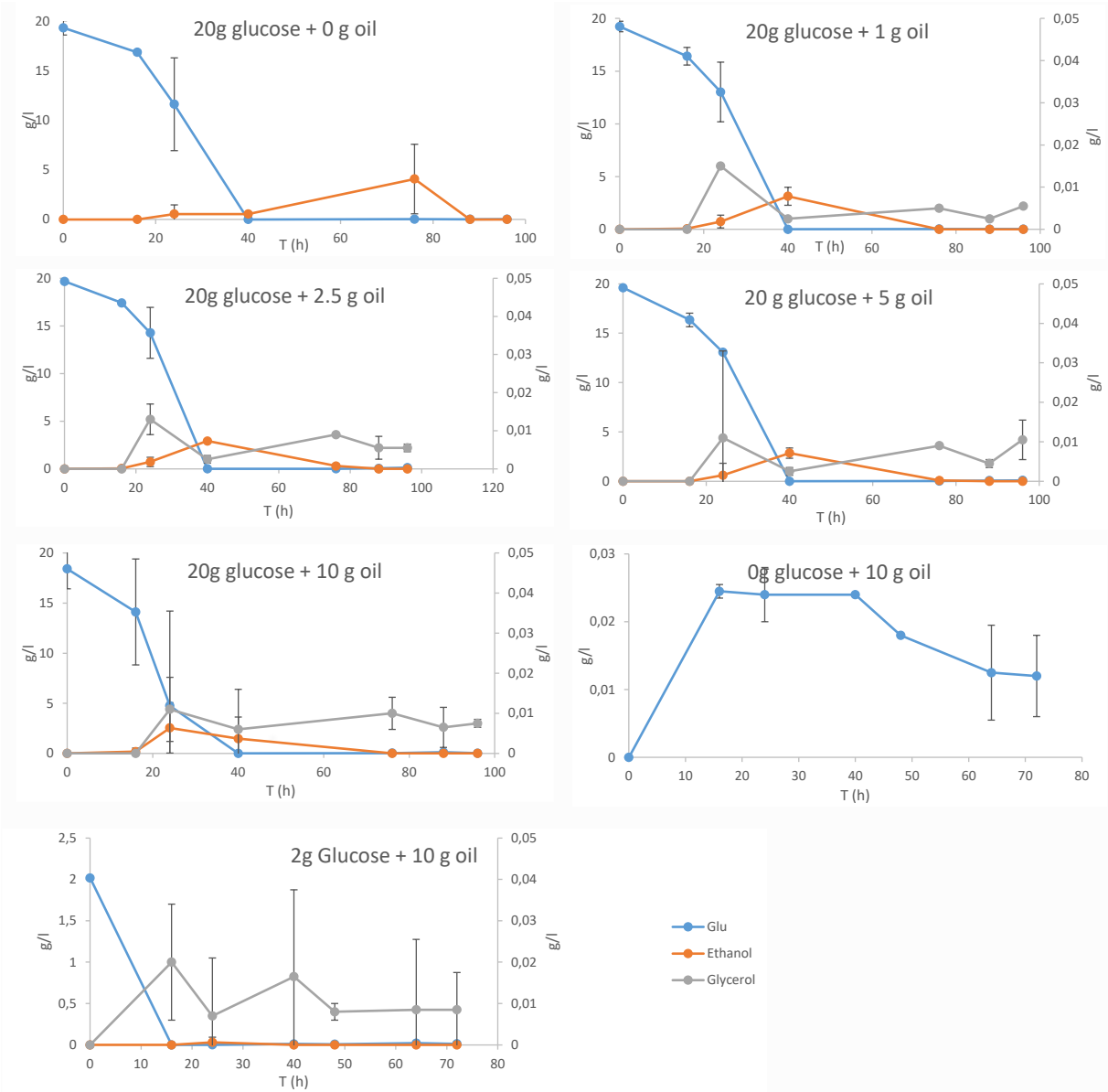


Figure 3. HPLC analysis of metabolites produced during fermentation

4.2 Effect of pH on oil assimilation by filamentous fungi

pH is one of the factors that affects microbial growth rate, enzyme production, production of fungal biomass and growth morphology (Zhou et al., 2000). So, ascomycetes (*Aspergillus oryzae*, *Fusarium venenatum*, and *Neurospora intermedia*) and zygomycetes (*Rhizopus oligosporus*, *Rhizopus oligosporus var. microsporus*, *Rhizopus oryzae* and RM4) were grown on two different pH levels in the medium recipe described in Table 2 (recipe 8). The only difference with the previous experiment is that, olive oil was added after 32 hours of cultivation on glucose because pre grown biomass can consume oil more efficiently; the initial pH was adjusted at 5.5. *Fusarium venenatum* among the ascomycetes did not grow into biomass after 32 hours of cultivation and therefore the experiment was stopped. After 32 hours of cultivation the pH of *Aspergillus oryzae* and *Neurospora intermedia* cultivations was adjusted to 5.8 and 6.5 together with the addition of 10 g/l olive oil. For *R. oryzae* pH was adjusted to 5.8 and 6.5 with the addition of 10 g/l olive oil after 57 hours of cultivation because the glucose consumption by the fungus was completed around 57 hours (Table 5). Dry biomass weight was measured after 32 hours of cultivation (*A. oryzae*, *N. intermedia*), 57 hours of cultivation with glucose (*R. oryzae*) and at the end of experiment i.e. 100 hours (*A. oryzae*, *N. intermedia*) and 125 hours (*R. oryzae*) of cultivation (Fig 4). *Aspergillus oryzae* produced biomass with a yield of 46.5% and 48.5% at pH 6.5 and 5.8, respectively, *Neurospora intermedia* produced biomass with a yield of 22.6% and 25.2% at pH 5.8 and 6.5, respectively, while *R. oryzae* produced biomass with a yield of 21% and 25 % at pH 5.8 and 6.5, respectively. *A. oryzae* produced more than the double the amount of biomass produced by *N. intermedia* and *R. oryzae* which indicates comparatively superior capability of growth and resistance to the inhibitors present in olive oil (Fig 4).

Table 5. Cultivation media characteristics

	Recipe table 2	Fungal strain	Glucose (g/l)	Olive oil (g/l) Addition after 32h	pH after 32 hours (Initial pH 5.5)	pH adjustment after 32 hours	Final pH
Ascomycetes	Recipe 8	<i>A. oryzae</i> ,	20	10	5.8	5.8	7.3
						6.5	7.6
	Recipe 8	<i>N. inter</i>	20	10	6.4	5.8	6.3
						6.5	7.2
		Fungal strain	Glucose (g/l)	Olive oil (g/l) Addition after 57h	pH after 57 hours (Initial pH 5.5)	pH adjustment after 57 hours	Final pH
Zygomycetes	Recipe 8	<i>R. oryzae</i>	20	10	6.1	5.8	7.09
						6.5	7.0

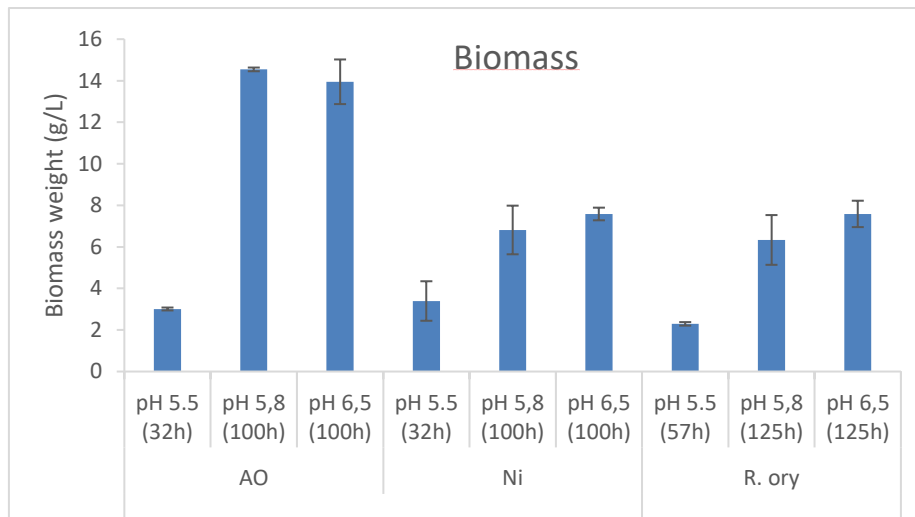


Fig 4. Dry biomass weight for ascomycetes (*A. oryzae* and *Neurospora intermedia*) and zygomycetes (*R. oryzae*)

The experiments mentioned above were carried out with pH adjustment to 5.5 before sterilization; it was observed that the pH dropped to 4.1 after sterilization. Contrary to *A. oryzae* and *Neurospora intermedia*, the zygomycetes did not grow in the media with pH adjustment before autoclave. The experiments using zygomycetes were then repeated using the same recipe but the pH adjustment was carried out after the sterilization. *Rhizopus oryzae* was the only fungus that grew into biomass among the four tested strains of zygomycetes (*Rhizopus oligosporus*, *Rhizopus oligosporus* var. *microsporus*, *Rhizopus oryzae* and RM4). Thus, zygomycetes are more sensitive to pH. Accordingly, only the cultivation of *Rhizopus oryzae* that grew into biomass followed the same experimental strategy as mentioned above; after 57 hours of cultivation, oil was added with pH adjustment at 5.8 and 6.5, in comparison to ascomycetes that was added after 32 hours of cultivation (Table 4). The experiment was stopped after 125 hours and dry biomass weight was measured which showed a yield of 21% and 25% at pH 5.8 and 6.5, respectively (Fig 4). *A. oryzae* had superior biomass growth rate than other species e.g. *N. intermedia* and *R. oryzae* when grown on spent sulphite liquor and wheat-based thin stillage (Asadollahzadeh et al., 2018, Ferreira et al., 2014b). *A. oryzae* was selected for further research study due to the flexibility of growing in the vast range of pH, culture conditions and presence of inhibitors.

For identification of metabolites, HPLC was performed and the results are presented in Fig 5. *Aspergillus oryzae* and *N. intermedia* consumed glucose after around 32 hours of cultivation. When glucose becomes depleted in the medium the fungi start using metabolites such as ethanol and glycerol to produce biomass. In another study done by Taherzadeh et al. (2003) 20 g/L glucose were consumed within the same period of time (32 h) when grown in synthetic medium

and paper pulp spent sulfite liquor (SSL) by *R. oryzae*. The ethanol produced was also consumed by *Aspergillus oryzae* after around 50 and 80 hours of cultivation at pH 6.5 and 5.8, respectively. Glycerol was detected after 32 hours of cultivation and increased over time after ethanol assimilation by fungi, pointing out consumption of olive oil by the fungi. *N. intermedia* consumed glucose within the same timeframe as *Aspergillus oryzae* i.e. after around 32 hours of cultivation while *Neurospora intermedia* consumed ethanol at a different rate. Glycerol was detected at 32 h and 38 h of cultivation and was consumed quickly by *Neurospora intermedia*. *R. oryzae* takes more time to consume glucose (75 hours) than *A. oryzae* and *N. intermedia*. The ethanol and glycerol concentration increased over time (25 – 75 hours) was not consumed completely until the end of cultivation i.e. 125 hours (Fig 5).

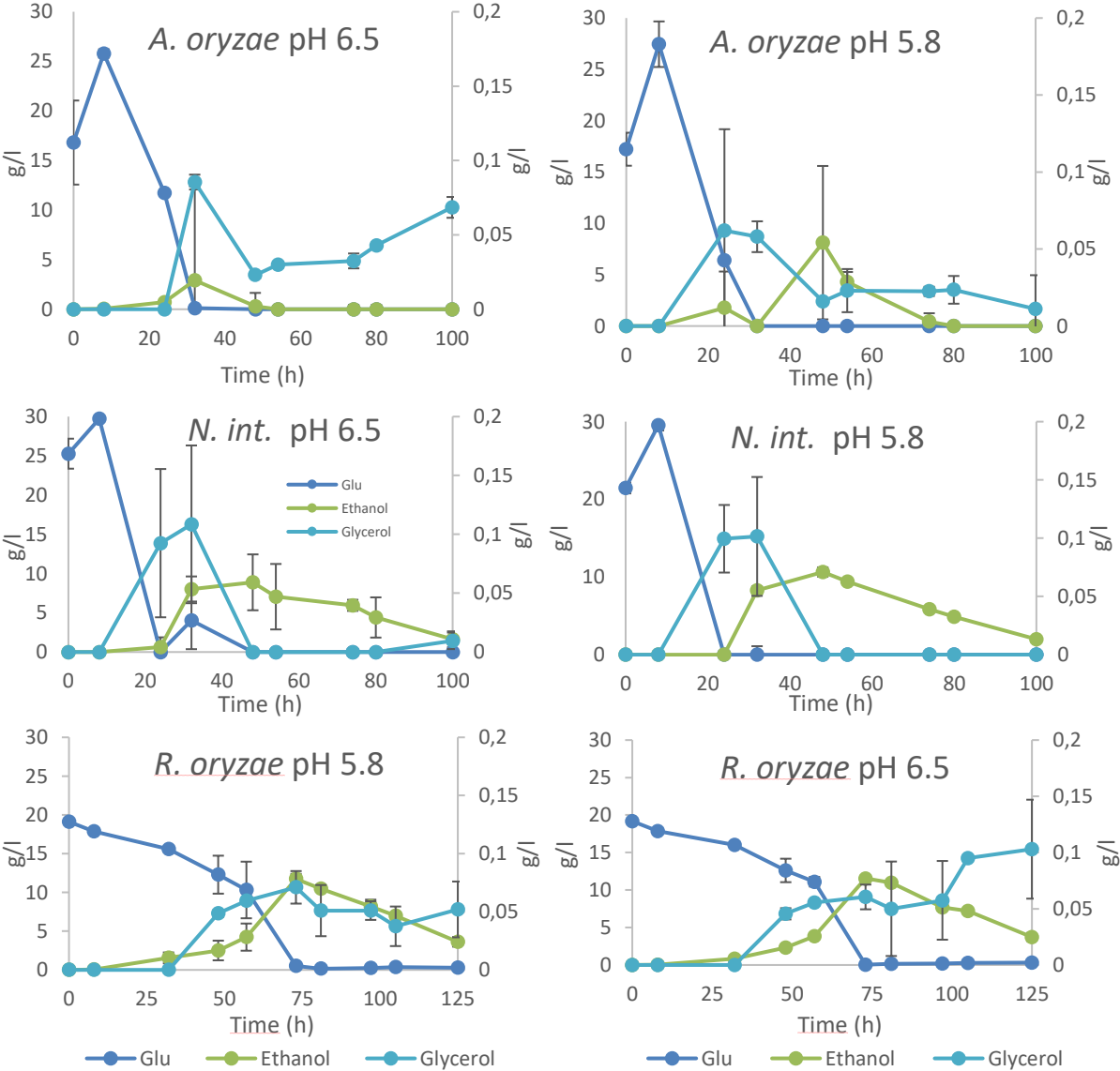


Figure 5. HPLC analysis of *A. oryzae*, *N. intermedia* and *R. oryzae* metabolites produced during cultivation.

4.3. Effect of oil concentration

Carbon is the major source of carbon and energy for fungi; however other nutrients are needed such as vitamins and minerals. Olive oil is a potential carbon source for fungi since it is composed of 60% carbon (van Nieuwenhuijzen et al., 2019). The effect of different oil concentrations has been tested on the growth of *A. oryzae*. The fungal strain was first grown in glucose as a sole carbon source. After 32 hours, different concentrations of olive oil, namely 10, 20, 40, 60, 80, and 100 g/L were added to observe the effect on the growth of the filamentous fungus. The amount of fungal biomass increased with increasing oil concentration up to 40 g/L. The decrease in biomass production observed after 40 g/L is possibly due to the lack or depletion of oxygen by thick layer interface present between oil and aqueous solution, inhibiting oxygen diffusion (Fig 6).

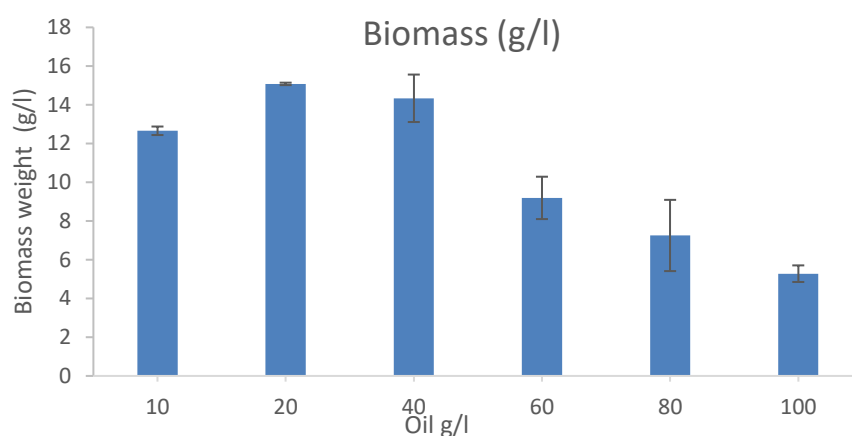


Fig 6. Effect of oil concentration on *A. oryzae* biomass growth.

HPLC was also performed on the samples taken at different intervals during cultivation of *A. oryzae* at different oil concentrations. Glucose was completely utilized by the fungus within 30 to 40 hours of cultivation with production of ethanol. The increase in glycerol production to 0.05 g/L was observed with the decrease in ethanol concentration at later stage of cultivation (Fig 7).

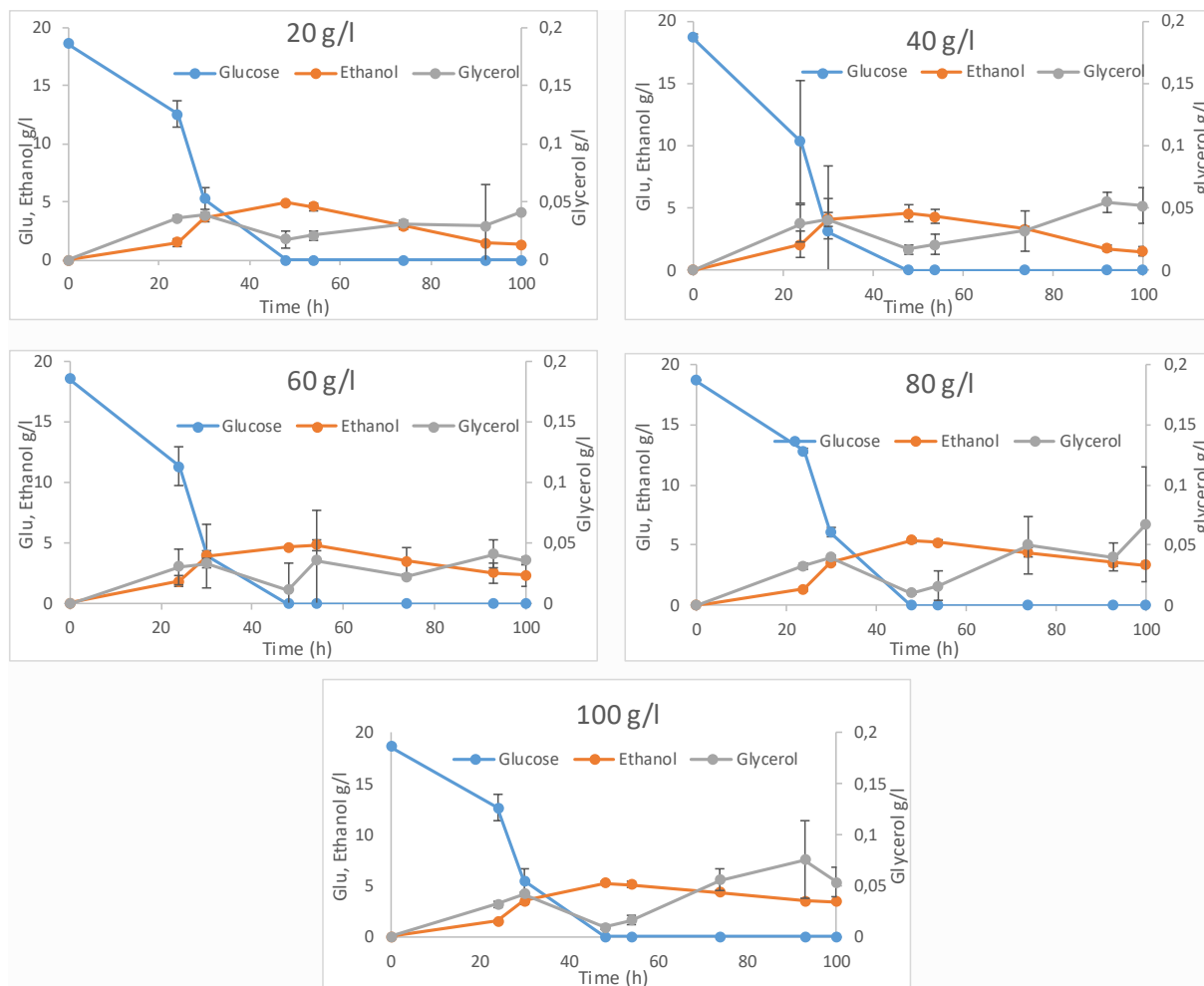


Fig 7. HPLC results, effect of different oil concentration on *A. oryzae*

4.4. Effect of glucose and glycerol on oil assimilation of *A. oryzae*

As there were no signs of glycerol concentration equivalent to the total oil degradation, *A. oryzae* growth behavior was investigated by growing the fungal strain in different sources of carbon as follows: i) Sole growth in glycerol as carbon source, ii) Glucose and glycerol as carbon sources and iii) glucose, glycerol and olive oil as carbon sources. It was observed that 7 g/L glycerol were consumed by the fungus within 80 hours of cultivation and it was completely consumed within 60 hours if grown with glucose. A similar experiment was done by Mahboubi et al. (2017), in which 28 g/L glycerol were consumed within 96 hours in the presence of yeast extract and only 2 g/L were consumed without using yeast extract. Different oil concentrations i.e. 5, 10, 20, 40 and 80 g/L and 12 g/L glycerol were added after 32 hours of cultivation in initially grown biomass in 20 g/L glucose. Results showed an initial increase in glycerol concentration of 0.8, 0.9, 1.9, 4.3 and 3.2 g/L with oil concentration of 5, 10, 20, 40 and 80 g/L, respectively (Fig 8A). Maximum biomass concentration of 11.7 g/L was obtained with 20 g/L concentration of olive oil (Fig 8B).

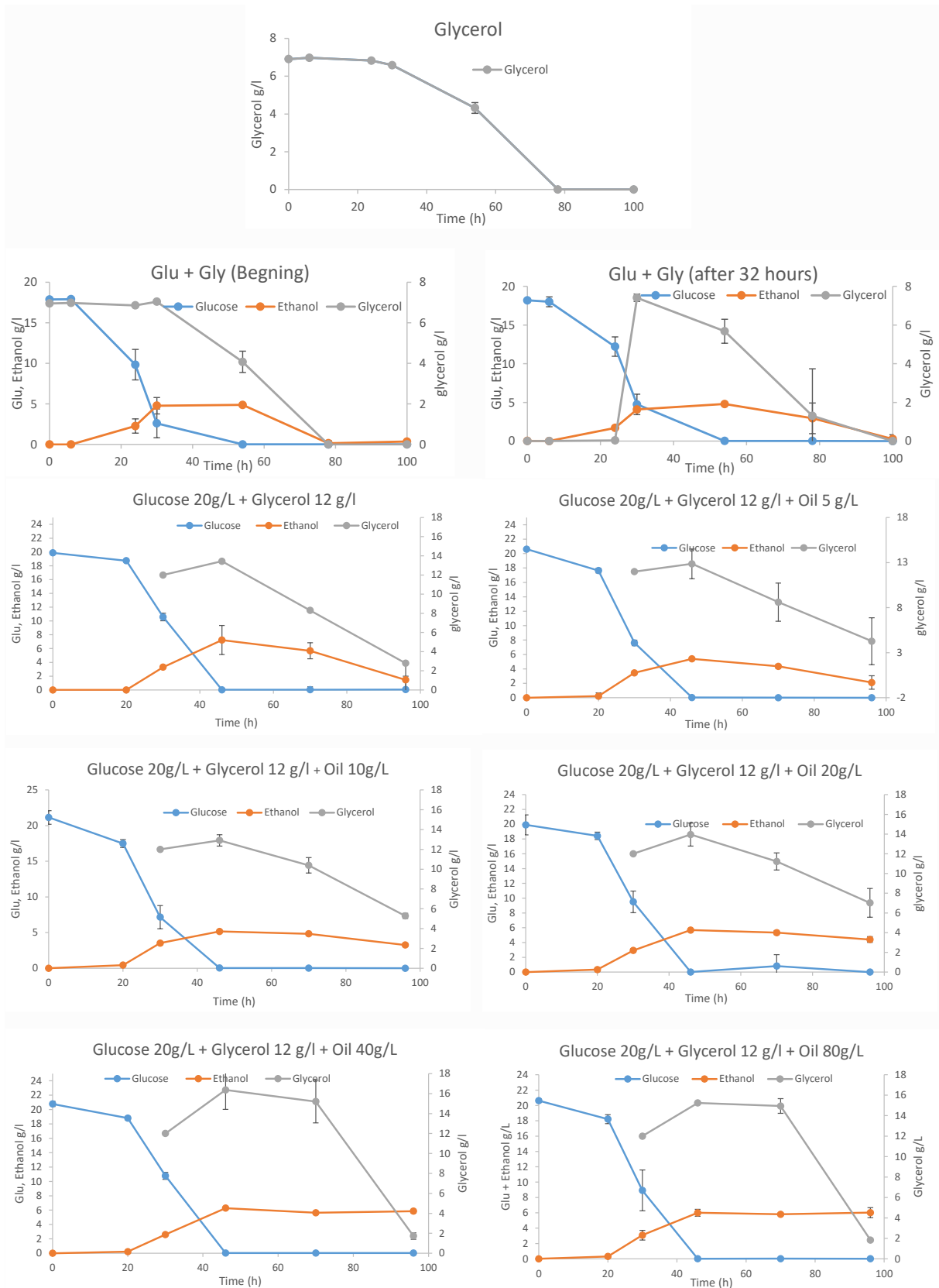


Fig 8A. HPLC results for effect of glycerol on *A. oryzae*

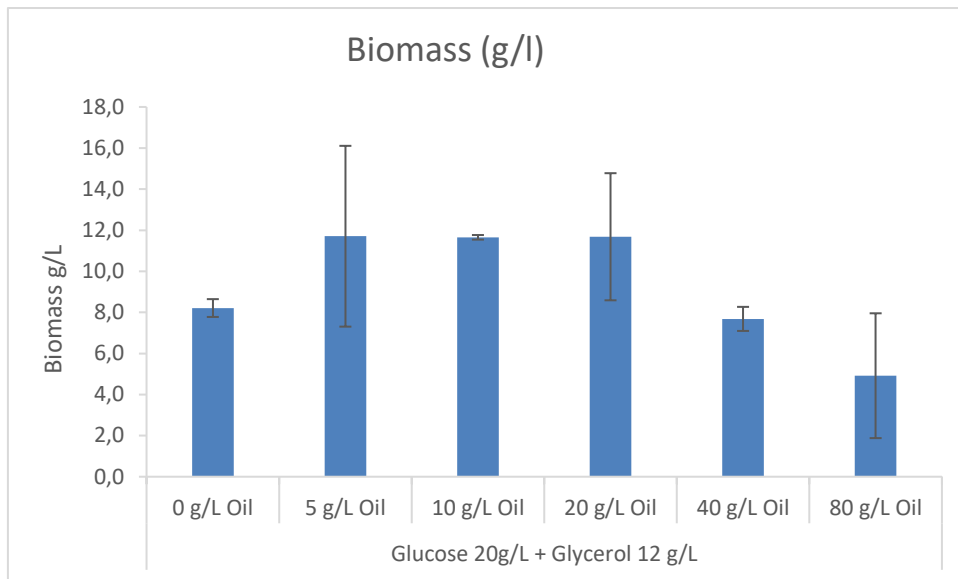


Fig 8B. Effect of glycerol on oil assimilation of *A. oryzae* biomass growth.

4.5. Biomass analysis

Fungal biomass was dried and compared with two different methods i.e. freeze dried and oven dried. Freeze dried process gave rise to slightly lower yield of *A. oryzae* biomass i.e. 1.25, 1.78, 2.0 and 2.5 g/L at oil concentration of 0, 10, 20 and 40 g/L in comparison to the biomass dried using the oven method (Fig 9A).

The protein content was measured using Kjeldahl method. *A. oryzae* grew better than *N. intermedia* in the absence of olive oil having protein yield of 48% and 36% (w/w) through freeze and oven dried method, respectively, and *N. intermedia* biomass was composed of 40% and 42% (w/w) protein using freeze and oven dried method, respectively (Fig 9A). Higher amounts of *A. oryzae* biomass produced (1.8, 12.7, 13.0 and 11.9 g/L) using different oil concentrations (0, 10, 20 and 40 g/L) had lower protein content, namely of 48%, 20%, 16% and 14% (w/w), respectively, whereas the corresponding fat contents were 3%, 12%, 32% and 32% (w/w), respectively. Ash contents were also measured, which showed decrease in ash contents (22%, 12%, 11%, and 10%) with increase in olive oil concentration (0, 10, 20 and 40 g/L) for *A. oryzae* (Fig 9B). Similar results were obtained for *N. intermedia* at 0 and 10 g/L of olive oil which showed biomass contents of 2.1 and 6.6 g/L with decreased protein contents of 40% and 30%, respectively. Increases in biomass fat contents were observed with the addition of oil which showed 3% and 9.8% fat with 0 and 10 g/L oil, respectively. Ash contents were 29% and 17% at 0 and 10 g/L of oil, respectively. Alkali insoluble materials, that represent the cell wall content of fungal biomass, were also measured, which showed 26, 46, 38 and 16% at increasing olive oil concentration (0, 10, 20, 40 g/L) for *A. oryzae*. For *N. intermedia*, AIM accounted to 25% and 17% of the dry weight of fungal biomass with 0 and 10 g/L of oil, respectively (Fig

9B). Normally, the fat contents of ascomycetes and zygomycetes are between 3 and 11% with the presence of 40% protein when using carbohydrates as a source of carbon (Asadollahzadeh et al., 2018). Using olive oil as a carbon source increased the fat contents up to 32%. Therefore, these results show the possibility to steer the protein and fat contents of fungal biomass by addition of olive oil which can diversify the range of potential applications in e.g. feed, food and biodiesel sectors.

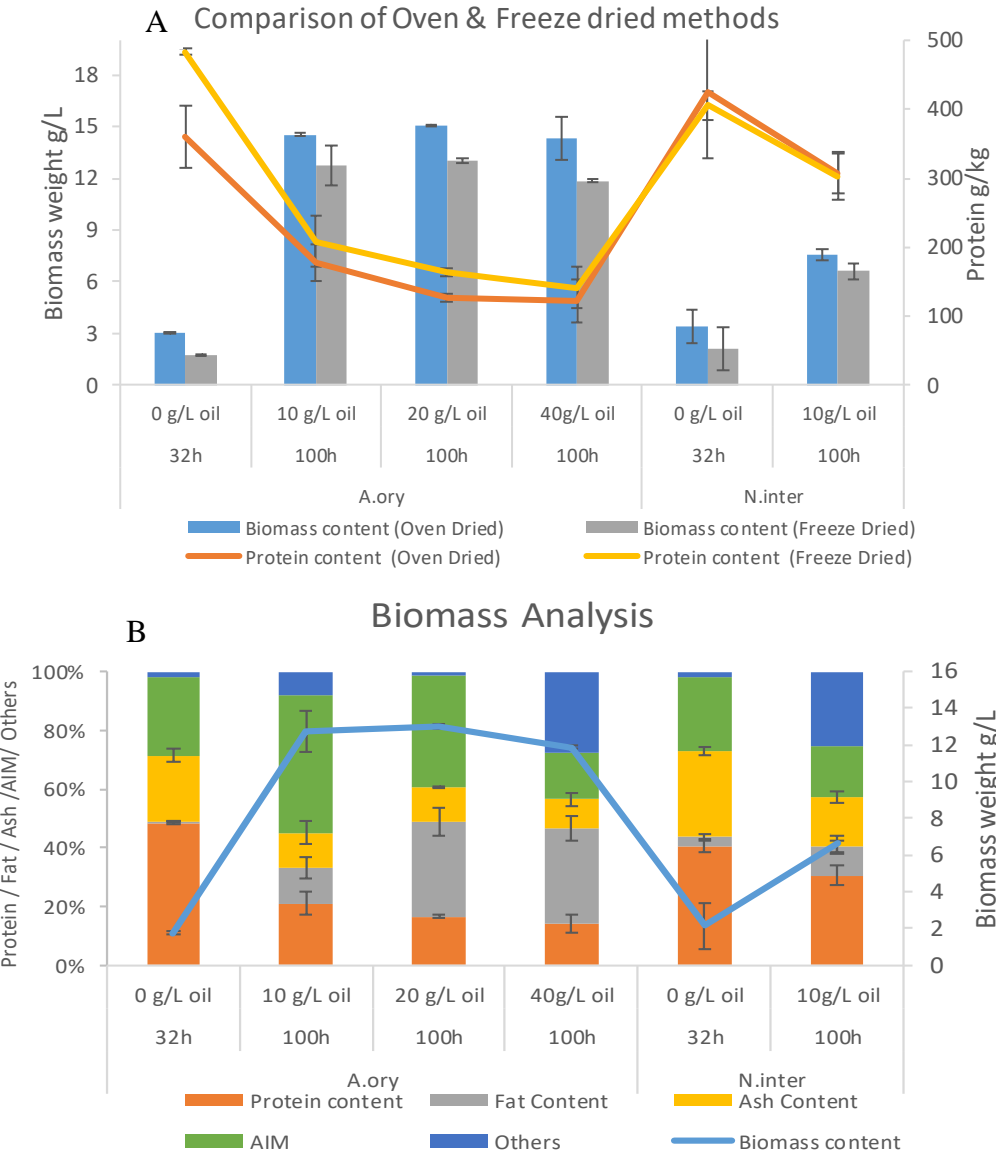


Fig 9. A: Comparison of biomass analysis by freeze and oven dried methods. B: Fat content, protein content, and biomass concentration analysis of freeze dried biomass.

4.6. Sudan Black Staining

Oil accumulation inside the fungal cells was studied using Sudan black staining. Fungal cells have significant capability of storing oil inside their mycelia (Papanikolaou et al., 2011). *A. oryzae* cells grown on 5 g/L oil were studied for Sudan black staining. Presence of blue black stains inside the fungal cells were indication of oil accumulation by fungal cells (Fig 10).

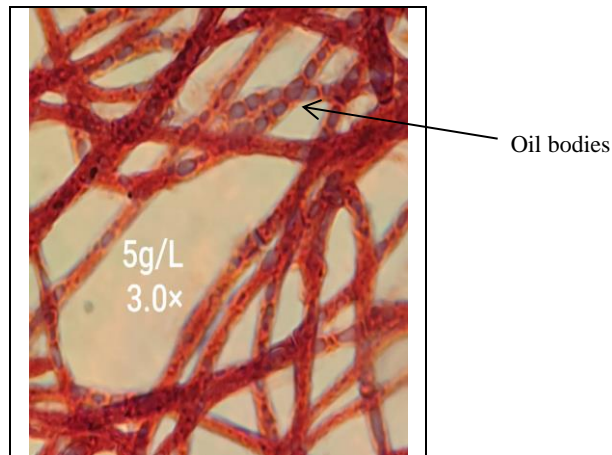


Fig 10. Sudan black stain of *A. oryzae*, grown on 5 g/L olive oil.

4.7. Effect of yeast extract on fungal biomass growth

Yeast extract (5g/L) was added to the cultivation medium instead of glucose. Fungal biomass concentrations of 0.7 g/L and 2.04 g/L were obtained when only yeast extract and yeast extract with nutrients were used as the cultivation medium (Table 3). With the addition and increase of oil concentration up to 100 g/L, the amount of biomass obtained was 13.21 g/L at 40 g/L of olive oil, which decreased at higher concentrations. Results are comparable with those obtained when a pre-germination stage in glucose was applied (Fig. 6), where a maximum amount of biomass of 14.33 g/L was obtained at 40 g/L of olive oil.

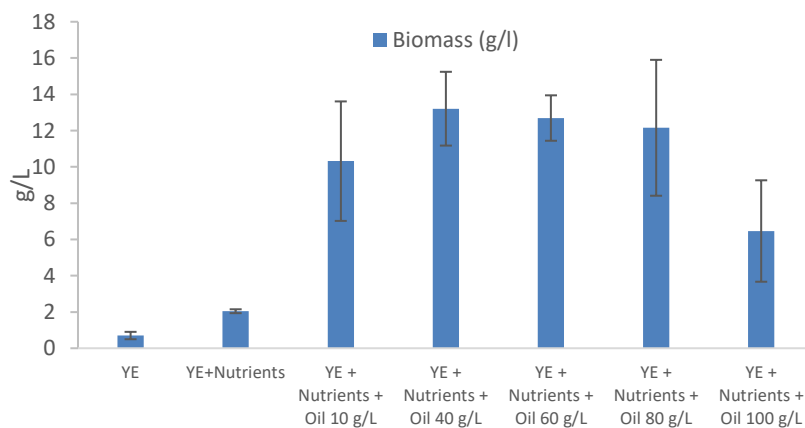


Fig 11. Effect of Yeast extract on fungal biomass growth

4.8. Effect of yeast extract concentration and incubation time

The effect of yeast extract concentration, namely of 0.5, 2.75, and 5 g/L was also investigated in the presence of 10 g/L of olive oil over an incubation time of 91 hours (Fig. 12A). Exponential phase was clearly seen until 66 hours of incubation followed by the stationary phase until 91 hours. The results unveiled a clear decrease in the fungal growth performance when yeast extract concentration was reduced by 50%, pointing out the need of further investigations on the optimization of its concentration per gram of olive oil. The growth performance of *A. oryzae* in 10 g/L of olive oil was also compared in the presence and absence of additional nutrients at yeast extract concentrations of 2.75 and 5 g/L (Fig 12B). Considering the results obtained, the addition of nutrients might offset the need of higher concentrations of yeast extract.

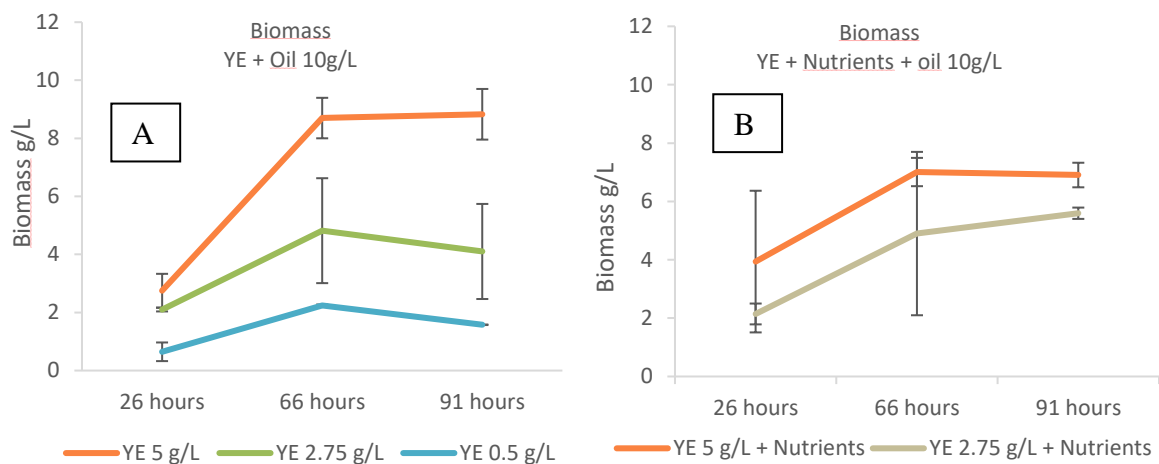


Fig 12. Fungal growth curve with different concentrations of yeast extract. A: Growth curve with YE. B: Growth curve with YE and additional nutrients.

4.9. Biomass Analysis

A. oryzae biomasses obtained using 5 g/L yeast extract with different concentrations of oil, namely 0, 10, 20 and 40 g/L were subject to compositional analysis, including protein, fat, ash, and cell wall contents. The compositional analysis of *A. oryzae* biomass grown in 20 g/L of glucose was used as reference for comparison. It is clearly observed from Figure 13 that an increase in olive oil content in the medium led to a decrease of the protein content from 46 to 14% and an increase in the fat contents from 4 to 34%. The contents of ash and alkali insoluble material also decreased with the increase in oil concentration, namely from 10 to 1% and from 25 to 12%, respectively. Another observation is that as the oil concentration in the medium increased, the amount of unknown compounds also increased which requires further studies.

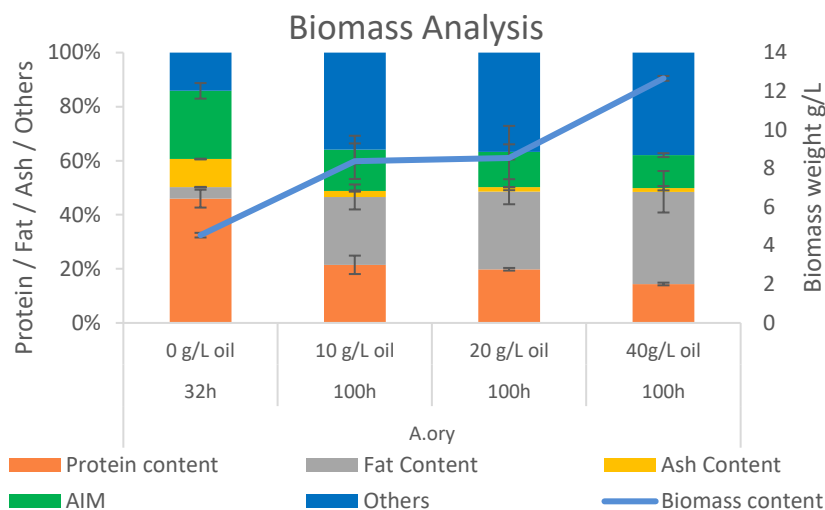


Fig 13. Biomass analysis of fungus grown using yeast extract as a nutrient media

5. Discussion

In this study, the growth of ascomycetes (*Aspergillus oryzae*, *Fusarium venenatum* and *Neurospora intermedia*) and zygomycetes (*Rhizopus oligosporus*, *Rhizopus oligosporus var. microsporus*, *Rhizopus oryzae* and *Rhizopus sp.*) in pure olive oil was studied.

Microorganisms such as fungi and algae have the capacity to produce lipases and store lipids in their cells through consumption of fat-rich substances. The produced and stored lipids in the cells of the microorganisms are very similar to those found in plant sources which are mainly composed of polyunsaturated fatty acids with great medicinal and nutritional importance (Papanikolaou and Aggelis, 2010).

Different growth media were investigated for the growth of filamentous fungi with olive oil as carbon source (Table 2 and 3), which revealed two strategies for successful fungal growth. One of such strategies includes the need of a pre-germination stage in a glucose-containing medium so that the filamentous fungi can then be grown in olive oil. This strategy can be further modified and lead to a shorter cultivation time through replacement of glucose by yeast extract, which enables fungal growth directly in olive oil. Other studies have also revealed the use of supplemented media for growth of microorganisms on oily substrates. Brozzoli et al. (2009b) studied biomass growth and lipase activity of *Candida cylindracea* on olive mill wastewater (OMW). The authors supplemented the OMW medium with 5 g/L glucose which lead to the production of 8.12 g/L biomass, while medium supplementation with yeast extract, peptone and olive oil lead of a biomass concentration of 8.59 g/L with lipase activity of 10 U/ml. In another study, *Candida cylindracea*, *Penicillium citrinum*, *Mucor hemalis*, and *Rhizopus oryzae* were

tested on palm oil effluent, where the authors used NH₄Cl, peptone, malt extract and olive oil rusts in the fungal growth recipe (Salihu et al., 2011).

In this study, three strains (*A. oryzae*, *N. intermedia* and *R. oryzae*) were grown into biomass in olive oil-containing medium among seven filamentous fungal strains tested. *A. oryzae* produced 94% more biomass than *N. intermedia* and *R. oryzae* (Fig 4). Four other filamentous fungi strains (*Conidiobolus nanodes*, *Entomophthora exitalis*, *Mortierella isabellina*, and *Mucor circinelloides*) were used in the study done by Kendrick and Ratledge (1996). These strains grown on various oils such as triolein, sesame, safflower and linseed produced 0.13 to 0.3 g/g of biomass. In comparison to Kendrick and Ratledge (1996), the maximum amount of biomass obtained using olive oil in this study with *A. oryzae* was 0.4 g/g.

Moreover, the produced biomass in this study was analyzed regarding protein, fat, ash, and cell wall (alkali-insoluble material) content. Using 20 g/L glucose and 10 g/L olive oil for the growth of *A. oryzae* originated a biomass yield of 42%. Increasing the olive oil concentration in the media to 20 g/L and 40 g/L decreased the *A. oryzae* biomass yield to 32% and 19%, respectively, possibly due to some fractions of oil remained unconsumed at the end of cultivation. There was a great increase in the *A. oryzae* biomass fat contents using olive oil as a substrate with a concomitant decrease in the protein contents; the fungal biomass contained 11.9%, 31.9% and 31.4% fat at 10, 20 and 40 g/L oil, respectively (Fig 9B). This concentration of fat in *A. oryzae* is much lower if carbon sources other than olive oil are used (Ozsoy et al., 2015, Subhash and Mohan, 2011) (Asadollahzadeh et al., 2018). Similar trends were found for *N. intermedia*, where the fat contents of its biomass were 10% and 22% using glucose and olive oil, respectively, pointing out its inferior growth performance in comparison to that of *A. oryzae*.

Similar trends were also found for *A. oryzae* biomass composition grown in olive oil with added yeast extract. Increase of oil concentration lead to significant decrease of the protein concentration from 45 to 14 % and increase of fat content to up to 34% (Fig 13). A comparison of the fat content of fungal biomass obtained in this study with that obtained in studies available in literature, using various filamentous fungal strains and substrates (Table 6), revealed that the range of fat concentrations are mostly lower than that reported in this study, with the exception of a few comparable values.

Table 6. comparison of fat contents in different filamentous fungi using different substrates

Fungi	Substrate	% Fat content	Source
<i>A. oryzae</i>	sugar beet pulp	4.5	(Ozsoy et al., 2015)
<i>A. oryzae</i>	corn cob waste liquor	22	(Subhash and Mohan, 2011)
<i>A. oryzae</i>	Spent sulfite liquor	3-11	(Asadollahzadeh et al., 2018)
<i>M. indicus</i>	Spent sulfite liquor	5	(Asadollahzadeh et al., 2018)
<i>R. oryzae</i>	Spent sulfite liquor	5	(Asadollahzadeh et al., 2018)
<i>A. oryzae</i>	vinasse	7	
<i>N. intermedia</i>	vinasse	3.5	(Karimi et al., 2019)
<i>R. oryzae</i>	vinasse	5.5	(Karimi et al., 2019)
<i>Lipomyces starkey</i>	OMW	29	(Yousuf et al., 2010)
<i>A. oryzae</i>	glucose	3	Current study
<i>A. oryzae</i>	Olive oil	34	Current study

Inversely, reductions in biomass protein contents were observed with the increase in biomass fat contents. Biomass protein contents decreased from 48% to 14% with the increase of fat contents to up to 31.9% from 0.3%. Similar results were obtained by Chan et al. (2018), who grew filamentous fungi (*Mucor circinelloides*) on whey which produced biomass with 24% oil contents and 20% protein contents.

In the current investigation, the growth of filamentous fungi in pure olive oil was investigated. *A. oryzae* was found to be the best among other strains of ascomycetes and zygomycetes (*N. intermedia* and *R. oryzae*) tested and grew well up to an olive oil concentration of 40 g/L. The insights produced by the present research can potentially be applied on olive oil based waste streams, where the growth of filamentous fungi can lead to the remediation of the olive mill wastewater with concomitant production of value-added products. This hypothesis is based on the fact that olive mill wastewater possesses 1% to 10% of olive oil (Azbar et al., 2004). However, it is also hypothesized that different outputs can be obtained in view of the dissimilar composition of olive mill wastewater in comparison to medium recipes used in this study. In addition to oil, olive mill wastewater has e.g. volatile and mineral solids, polyalcohol, pectin, tannins and polyphenols (Azbar et al., 2004).

Overall, the insights gathered in this thesis work opens the possibility for steering the growth of filamentous fungi with olive oil, which, in turn, can facilitate the genesis of processes tailored to the final application of fungal biomass in feed, food and biofuel production.

Proposals for future research include the study on the need of enzyme addition to the medium to facilitate oil degradation and consequent fungal growth. This can potentially also increase the olive oil concentration that can be added to the medium without impairment of fungal growth performance. Furthermore, a comprehensive analysis of fat present inside of fungal cells and left in the cultivation medium is of utmost importance in order to provide an understanding of the mechanism behind oil removal from the medium. Fungal cultivation should also be carried out in bioreactors providing efficient stirring and aeration in order to provide a thin-layer contact between oil droplets and fungal cells and enzymes.

6. Conclusion

The present study showed the steering effect of olive oil can have on the composition of *A. oryzae* biomass regarding fat and protein contents. At the best conditions found in this study, a biomass yield of 42% was obtained together with a biomass fat content of up to 34% on a dry weight basis. However, pre-germination of filamentous fungi in glucose or addition of complex nutrient sources such as yeast extract to the medium, were needed to attain successful fungal growth in olive oil-containing medium recipes. It is hypothesized that these needed strategies can be smoothed up by the use of external lipase or through cultivation in bioreactors with proper agitation and aeration. The cultivation strategies developed in this work can potentially be applied for the remediation of waste cooking oil and waste streams of olive oil industries, namely olive mill wastewater. The insights produced in this thesis work show the possibility of steering the level of fat and protein in fungal biomass via control of olive oil concentration that in turn, can diversify the range of applications of fungal biomass in feed, food and biofuel sectors. This, in turn, increases the interest of the use of fungal cultivation for recovery of nutrients from oil-rich waste streams in the form of value-added products.

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References

- ANDUALEMA, B. & GESSESSE, A. J. B. 2012. Microbial lipases and their industrial applications. *Biotechnology*, 11, 100-118.
- ARACELI, T. H. & HUGO, L. 2019. The Role of the Filamentous Fungi in Bioremediation. *Fungal Bioremediation*. Taylor and francis group.
- ARVANITTOYANNIS, I. S. & LADAS, D. 2008. Meat waste treatment methods and potential uses. *International Journal of Food Science and Technology*, 43, 543-559.
- ASADOLLAHZADEH, M., GHASEMIAN, A., SARAEIAN, A., RESALATI, H. & TAHERZADEH, M. J. 2018. Production of Fungal Biomass Protein by Filamentous Fungi Cultivation on Liquid Waste Streams from Pulping Process. *Bioresources*, 13, 5013-5031.
- AZBAR, N., BAYRAM, A., FILIBELI, A., MUEZZINOGLU, A., SENGUL, F. & OZER, A. 2004. A review of waste management options in olive oil production. *Critical Reviews in Environmental Science and Technology*, 34, 209-247.
- BEDNARSKI, W., KOWALEWSKA-PIONTAS, J., ZEGARSKA, Z. & ADAMCZAK, M. 1993. Growth of three fungi on poultry fat or beef tallow. *World J Microbiol Biotechnol*, 9, 656-9.
- BONDIOLI, P., CARELLI, G. P. & GROSSO, M. 2019. Animal fats for non-food uses. A review of technology and critical points. *Rivista Italiana Delle Sostanze Grasse*, 96, 5-15.
- BOYD, L. C., DRYE, N. C. & HANSEN, A. P. 1999. Isolation and characterization of whey phospholipids. *Journal of Dairy Science*, 82, 2550-2557.
- BROZZOLI, V., CROGNALE, S., SAMPEDRO, I., FEDERICI, F., D'ANNIBALE, A. & PETRUCCIOLI, M. 2009a. Assessment of olive-mill wastewater as a growth medium for lipase production by *Candida cylindracea* in bench-top reactor. *Bioresource Technology*, 100, 3395-3402.
- BROZZOLI, V., CROGNALE, S., SAMPEDRO, I., FEDERICI, F., D'ANNIBALE, A. & PETRUCCIOLI, M. 2009b. Assessment of olive-mill wastewater as a growth medium for lipase production by *Candida cylindracea* in bench-top reactor. *Bioresource Technology*, 100, 3395-3402.
- BURDON, K. L. 1946. Fatty Material in Bacteria and Fungi Revealed by Staining Dried, Fixed Slide Preparations. *J Bacteriol*, 52, 665-78.
- CHAN, L. G., COHEN, J. L., OZTURK, G., HENNEBELLE, M., TAHA, A. Y. & BELL, J. M. L. N. D. 2018. Bioconversion of cheese whey permeate into fungal oil by *Mucor circinelloides*. *Journal of Biological Engineering*, 12.

- CHEN, W. P., HE, Y., ZHOU, Y. X., SHAO, Y. C., FENG, Y. L., LI, M. & CHEN, F. S. 2015. Edible Filamentous Fungi from the Species *Monascus*: Early Traditional Fermentations, Modern Molecular Biology, and Future Genomics. *Comprehensive Reviews in Food Science and Food Safety*, 14, 555-567.
- COLLA, L. M., FICANHA, A. M. M., RIZZARDI, J., BERTOLIN, T. E., REINEHR, C. O. & COSTA, J. A. V. 2015. Production and Characterization of Lipases by Two New Isolates of *Aspergillus* through Solid-State and Submerged Fermentation. *Biomed Research International*.
- CRINI, G. & LICHTFOUSE, E. 2019. Advantages and disadvantages of techniques used for wastewater treatment. *Environmental Chemistry Letters*, 17, 145-155.
- CROGNALE, S., D'ANNIBALE, A., FEDERICI, F., FENICE, M., QUARATINO, D. & PETRUCCIOLI, M. 2006. Olive oil mill wastewater valorisation by fungi. *Journal of Chemical Technology and Biotechnology*, 81, 1547-1555.
- DEWI, R., KASIAMDARI, R., MARTANI, E. & PURWESTRI, Y. Efficiency of *Aspergillus* sp. 3 to reduce chromium, sulfide, ammonia, phenol, and fat from batik wastewater. IOP Conference Series: Earth and Environmental Science, 2019. IOP Publishing, 012003.
- DIALLINAS, G., RAFAILIDOU, N., KALPAKTSI, I., KOMIANOU, A. C., TSOUVALI, V., ZANTZA, I., MIKROS, E., SKALTSOUNIS, A. L. & KOSTAKIS, I. K. 2018. Hydroxytyrosol (HT) Analogs Act as Potent Antifungals by Direct Disruption of the Fungal Cell Membrane. *Frontiers in Microbiology*, 9.
- FERREIRA, J. 2015. *Integration of filamentous fungi in ethanol dry-mill biorefinery*. Högskolan i Borås.
- FERREIRA, J. A., LENNARTSSON, P. R. & TAHERZADEH, M. J. 2014a. Production of Ethanol and Biomass from Thin Stillage Using Food-Grade Zygomycetes and Ascomycetes Filamentous Fungi. *Energies*, 7, 3872-3885.
- FERREIRA, J. A., LENNARTSSON, P. R. & TAHERZADEH, M. J. 2014b. Production of Ethanol and Biomass from Thin Stillage Using Food-Grade Zygomycetes and Ascomycetes Filamentous Fungi. Högskolan i Borås, Institutionen Ingenjörshögskolan.
- FERREIRA, J. A., MAHBOUBI, A., LENNARTSSON, P. R. & TAHERZADEH, M. J. 2016. Waste biorefineries using filamentous ascomycetes fungi: Present status and future prospects. *Bioresource Technology*, 215, 334-345.
- FRANKE-WHITTLE, I. H. & INSAM, H. 2013. Treatment alternatives of slaughterhouse wastes, and their effect on the inactivation of different pathogens: A review. *Critical Reviews in Microbiology*, 39, 139-151.
- GUN, I. & SIMSEK, B. 2011. The fatty acid composition of butter stored in sheep's or goat's stomach (Karinyagi). *Food and nutrition sciences*, 2, 402.
- JAYATHILAKAN, K., SULTANA, K., RADHAKRISHNA, K. & BAWA, A. S. 2012. Utilization of byproducts and waste materials from meat, poultry and fish processing industries: a review. *Journal of Food Science Technology*, 49, 278-293.
- KALAMBURA, S., VOĆA, N., KRIČKA, T., ŠINDRAK, Z., ŠPEHAR, A. & KALAMBURA, D. 2011. High-risk biodegradable waste processing by alkaline hydrolysis. *Archives of Industrial Hygiene Toxicology*, 62, 249-253.
- KARIMI, S., SOOFIANI, N. M., LUNDH, T., MAHBOUBI, A., KIESSLING, A. & TAHERZADEH, M. J. 2019. Evaluation of Filamentous Fungal Biomass Cultivated on Vinasse as an Alternative Nutrient Source of Fish Feed: Protein, Lipid, and Mineral Composition. *Fermentation-Basel*, 5.

- KENDRICK, A. & RATLEDGE, C. 1996. Cessation of polyunsaturated fatty acid formation in four selected filamentous fungi when grown on plant oils. *Journal of the American Oil Chemists Society*, 73, 431-435.
- KUMAR, D. S. & RAY, S. 2014. Fungal lipase production by solid state fermentation-an overview. *Journal of Analytical Bioanalytical Techniques*, 6, 1-10.
- LEOCI, R. 2014. *Animal by-products (ABPs): origins, uses, and European regulations*, Universitas Studiorum.
- MAHBOUBI, A., FERREIRA, J. A., TAHERZADEH, M. J. & LENNARTSSON, P. R. 2017. Production of Fungal Biomass for Feed, Fatty Acids, and Glycerol by *Aspergillus oryzae* from Fat-Rich Dairy Substrates. *Fermentation-Basel*, 3.
- MAJDEJABBARI, S., BARGHI, H. & TAHERZADEH, M. J. 2011. Synthesis and properties of a novel biosuperabsorbent from alkali soluble *Rhizomucor pusillus* proteins. *Applied Microbiology and Biotechnology*, 92, 1171-1177.
- MATTOS, R., STAPLES, C. R., ARTECHE, A., WILTBANK, M. C., DIAZ, F. J., JENKINS, T. C. & THATCHER, W. W. 2004. The Effects of Feeding Fish Oil on Uterine Secretion of PGF₂ α , Milk Composition, and Metabolic Status of Periparturient Holstein Cows. *Journal of Dairy Science*, 87, 921-932.
- NEMA, A., PATNALA, S. H., MANDARI, V., KOTA, S. & DEVARAI, S. K. 2019. Production and optimization of lipase using *Aspergillus niger* MTCC 872 by solid-state fermentation. *Bulletin of the National Research Centre*, 43, 82.
- NIETO, L. M., HODAIFA, G., RODRIGUEZ, S., GIMENEZ, J. A. & OCHANDO, J. 2011. Degradation of organic matter in olive-oil mill wastewater through homogeneous Fenton-like reaction. *Chemical Engineering Journal*, 173, 503-510.
- OZSOY, H. D., ARIKAN, E. B., CINKIR, C., ERYILMAZ, G. D., KUCUK, D. & VAN LEEUWEN, J. H. 2015. Fungal oil production from oleaginous fungi *Mucor circinelloides* and *Aspergillus oryzae* cultivated on sugar beet pulp. *APJES*, 3, 735-741.
- PAPANIKOLAOU, S. & AGGELIS, G. 2010. *Yarrowia lipolytica*: A model microorganism used for the production of tailor-made lipids. *European Journal of Lipid Science and Technology*, 112, 639-654.
- PAPANIKOLAOU, S., DIMOU, A., FAKAS, S., DIAMANTOPOULOU, P., PHILIPPOUSSIS, A., GALIOTOU-PANAYOTOU, M. & AGGELIS, G. 2011. Biotechnological conversion of waste cooking olive oil into lipid-rich biomass using *Aspergillus* and *Penicillium* strains. *Journal of Applied Microbiology*, 110, 1138-1150.
- PHAM, T. P., KAUSHIK, R., PARSHETTI, G. K., MAHMOOD, R. & BALASUBRAMANIAN, R. 2015. Food waste-to-energy conversion technologies: current status and future directions. *Waste Manag*, 38, 399-408.
- RYAN, M. & WALSH, G. 2012. The Characterisation of dairy waste and the potential of whey for industrial fermentation. Environmental Protection Agency.
- SALIHU, A., ALAM, M. Z., ABDULKARIM, M. I. & SALLEH, H. M. 2011. Suitability of using palm oil mill effluent as a medium for lipase production. *African Journal of Biotechnology*, 10, 2044-2052.
- SANKARAN, S., KHANAL, S. K., JASTI, N., JIN, B., POMETTO, A. L. & VAN LEEUWEN, J. 2010. Use of Filamentous Fungi for Wastewater Treatment and Production of High Value Fungal Byproducts: A Review. *Critical Reviews in Environmental Science and Technology*, 40, 400-449.
- SARANTOPOULOS, I., CHATZISYMEON, E., FOTEINIS, S. & TSOUTSOS, T. 2014. Optimization of biodiesel production from waste lard by a two-step transesterification process under mild conditions. *Energy for Sustainable Development*, 23, 110-114.

- SELVAM, D. J. P. & VADIVEL, K. 2012. Performance and emission analysis of DI diesel engine fuelled with methyl esters of beef tallow and diesel blends. *International Conference on Modelling Optimization and Computing*, 38, 342-358.
- SIBIRNY, A. A. 2017. *Biotechnology of Yeasts and Filamentous Fungi*, Springer, Cham.
- SLUITER, A., HAMES, B., RUIZ, R., SCARLATA, C., SLUITER, J. & D., T. 2008. Determination of Ash in Biomass: Laboratory Analytical Procedure (LAP).
- SUBHASH, G. V. & MOHAN, S. V. 2011. Biodiesel production from isolated oleaginous fungi *Aspergillus* sp. using corncob waste liquor as a substrate. *Bioresource Technology*, 102, 9286-9290.
- TAHERZADEH, M. J., FOX, M., HJORTH, H. & EDEBO, L. 2003. Production of mycelium biomass and ethanol from paper pulp sulfite liquor by *Rhizopus oryzae*. *Bioresource Technology*, 88, 167-177.
- TOSCANO, L., MONTERO, G., STOYTICHEVA, M., GOCHEV, V., CERVANTES, L., CAMPBELL, H., ZLATEV, R., VALDEZ, B., PEREZ, C. & GIL-SAMANIEGO, M. 2013. Lipase Production through Solid-State Fermentation Using Agro-Industrial Residues as Substrates and Newly Isolated Fungal Strains. *Biotechnology & Biotechnological Equipment*, 27, 4074-4077.
- WALLACE, T., GIBBONS, D., O'DWYER, M. & CURRAN, T. P. 2017. International evolution of fat, oil and grease (FOG) waste management - A review. *Journal of Environmental Management*, 187, 424-435.
- VAN NIEUWENHUIJZEN, E. J., SAILER, M. F., VAN DEN HEUVE, E. R., RENSINK, S., ADAN, O. C. G. & SAMSON, R. A. 2019. Vegetable oils as carbon and energy source for *Aureobasidium melanogenum* in batch cultivation. *Microbiologyopen*, 8.
- VIJAYAN, G., SARAVANANE, R. & SUNDARARAJAN, T. 2017. Carbon footprint analyses of wastewater treatment systems in puducherry. *Computational Water, Energy, and Environmental Engineering*, 6, 281-303.
- YOUSUF, A., SANNINO, F., ADDORISIO, V. & PIROZZI, D. 2010. Microbial Conversion of Olive Oil Mill Wastewaters into Lipids Suitable for Biodiesel Production. *Journal of Agricultural and Food Chemistry*, 58, 8630-8635.
- ZHAO, K. N., XU, R., ZHANG, Y., TANG, H., ZHOU, C. B., CAO, A. X., ZHAO, G. Z. & GUO, H. 2017. Development of a novel compound microbial agent for degradation of kitchen waste. *Brazilian Journal of Microbiology*, 48, 442-450.
- ZHOU, Y., DU, J. X. & TSAO, G. T. 2000. Mycelial pellet formation by *Rhizopus oryzae* ATCC 20344. *Applied Biochemistry and Biotechnology*, 84-6, 779-789.



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