THE EFFECT OF DESTONING AND ENZYMATIC PRETREATMENTS ON THE BIOFUEL PRODUCTION FROM OLIVE CAKE

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Patrick Tai

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COMMITTEE MEMBERSHIP

TITLE:	The effect of destoning and enzymatic pretreatments on the biofuel production from olive cake
AUTHOR:	Patrick Tai
DATE SUBMITTED:	May 2018
COMMITTEE CHAIR:	Stephanie Jung, Ph.D. Professor, Food Science and Nutrition Department
COMMITTEE MEMBER:	Amanda Lathrop, Ph.D. Associate Professor, Food Science and Nutrition Department
COMMITTEE MEMBER:	Jennifer Carroll, Ph.D. Associate Professor, Chemistry and Biochemistry Department

ABSTRACT

The effect of destoning and enzymatic pretreatments on the biofuel production from olive cake

Patrick Tai

More than 16,000 tons of olive cake was produced in the United States in 2017. Olive cake is a by-product of olive oil extraction, which has limited animal feed potential, and poses an environmental threat when landfilled due to its high organic load and polyphenol content. This residue has potential for biofuel (bioethanol and biomethane) production because it is rich in polysaccharides such as pectin, hemicellulose, and cellulose. Yet, olive cake contains olive stones that can impede its conversion to biofuel. Therefore, two methods of destoning, centrifugation and screening by horizontal screw press, were first compared. Both methods removed an equal percentage of stones (95%), but centrifugation partitioned the majority (57 – 79%) of digestible solids (olive pulp) with the stones. Then, two strategies were compared to maximize both biomethane and bioethanol production; enzymatic conversion of insoluble to soluble carbohydrates and destoning by screening. After 30 days of anaerobic digestion at 35 °C, both the enzymatically pretreated and the destoned olive cakes produced similar amounts of methane (~295 mL CH_4/g VS), 42% more than the control (209.5 mL CH_4/g VS). The biogas produced was composed of 60-70% methane. A comparison of biomethane yields with a broad range of agricultural residues demonstrated olive cake's suitability for biomethane production. The digestate, residue from the anaerobic digestion, have high Kjeldahl nitrogen content (3.6%, db) and low polyphenol concentration (0.02 mg

GAE/g), which then qualify it as an ingredient for soil amendment. Ethanol production investigations showed that after 3 days of fermentation at 32 °C, only the destoned and enzymatically pretreated olive cake produced ethanol (1.3 mg/mL). Acetic acid, an inhibitor of ethanol production, was present in all samples broth, suggesting microbial contamination was present.

These results provide evidence that olive cake can be diverted from landfills to be converted into a biofuel. Sustainable pretreatments such as destoning and enzymatic pretreatment increase biomethane yield. The digestate created from the anaerobic digestion of olive cake can be used as a soil amendment, adding further value to olive cake.

Keywords*:* Enzymatic pretreatment; Anaerobic digestion; Olive cake; Biomethane; Bioethanol; Digestate; Olive stone

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LIST OF MOST COMMONLY USED ABBREVIATIONS

2PC = two-phase centrifuge

2POMW = two-phase olive mill waste

3PC = three-phase centrifuge

3POMW = olive cake, three-phase olive mill waste

ADF = acid detergent fiber

ADL = acid detergent lignin

BMP = biochemical methane potential

DOC = diluted olive cake

DDOC = destoned diluted olive cake

DEDOC = destoned and enzymatically pretreated olive cake

EDOC = enzymatically pretreated diluted olive cake

GAE = gallic acid equivalent

GHG = greenhouse gas

HSP = horizontal screw press

HPLC = high performance liquid chromatography

NADES = natural deep eutectic solvent

NDF = neutral detergent fiber

OMW = olive mill waste (olive cake and olive mill wastewater)

OMWW = olive mill wastewater

S:I = substrate inoculum ratio

SFE = supercritical fluid extraction

SMY = specific methane yield

TMY = theoretical methane yield

TS = total solids

VS = volatile solids

CHAPTER 1 – INTRODUCTION

1.1 Background information

Olive oil is considered one of the world's oldest and most nutritious oils (Vossen, 2007). Over the centuries, olive oil has changed from a staple food to a luxury and now even a functional food due to its health benefits including, but not limited to, improvement of cardiometabolic markers, a reduction in cancer prevalence, and decreased instances of cardiovascular disease (Salas-Salvadó et al., 2008; Psaltopoulou et al., 20011). As global consumption of olive oil continues to increase, doubling from 2005 to 2017, global olive oil production and the production of olive mill wastes (OMW), solid and liquid residues from modern olive oil production, has also increased (International Olive Council, 2017). Industrial olive oil production relies on centrifuges, which separate the oil from the pulp, pits, skins, and water. One type of centrifuge used for olive oil production is the three-phase centrifuge. These centrifuges require the addition of 0.5 to 1 m^3 of water for every ton of olives processed, and produce two effluent streams, a solid olive cake, which is also known as three-phase olive mill waste (3POMW), and wastewater (OMWW). Disposal of these wastes poses great environmental threats to microbial communities in the soil, river ecosystems, and air quality (DellaGreca et al., 2001; Rana et al., 2003; Mekki et al., 2006)

The United States (US) is the third largest olive oil consumer in the world (accounting for 17% of all imports), but domestic production of olive oil only meets 3% of internal demand, leaving much room and desire to grow (United States International Trade Commision., 2013). California is the sole (> 99%) producer of olive oil in the US, with 15,000 tons produced in 2017, which generated approximately 76,000 tons of OMW

(Azbar et al., 2004; International Olive Council, 2017). In an attempt to reduce the amount of the OMW being land-filled, there has been a recent shift from trying to merely treat and then dispose this waste to valorizing it: extracting or manufacturing value-added products from agricultural residues.

In 2015, California used approximately 7.5 quads of energy with 75% of this energy coming from non-renewable fossil fuels, while only 11% came from renewable sources, including biomass (Fig. 1.1) (EIA, 2017). Because of climate change and the state's increasing energy needs, carbon neutral energy sources are necessary to replace the world's rapidly depleting supply of fossil fuel. Due to the high caloric value of olive cake (21 kJ/g TS), it is commonly burned for energy in Mediterranean countries. However, the new "California Global Warming Solutions Act of 2006: emissions limit" (approved September 2016) requires a 40% reduction in current greenhouse gas (GHG) emissions by 2030 (Pavely, 2016). In the future, burning agricultural residues such as olive cake to recover energy may not be permitted. One alternative method of extracting energy from agricultural residues without directly incinerating the substrate is the production of biofuels (Roig et al., 2006).

Biofuels such as bioethanol, biogas, and biodiesel, which are created from energy dense agricultural residues, are regarded as a more sustainable energy source as the biomass sequesters the carbon dioxide in the atmosphere, resulting in a carbon neutral fuel (Lal, 2005). The feedstock for first-generation biofuels, like corn or grain, require cropland. Many ecosystems have been disturbed and deforested in an attempt to grow more fuel, increasing GHG emissions (Fargione et al., 2008). Olive cake is a potential feedstock for second-generation biofuel as it is a waste product that does not require land-

use change. Therefore, using OMW for biofuel production not only adds value to the olive mill wastes but also helps the environment.

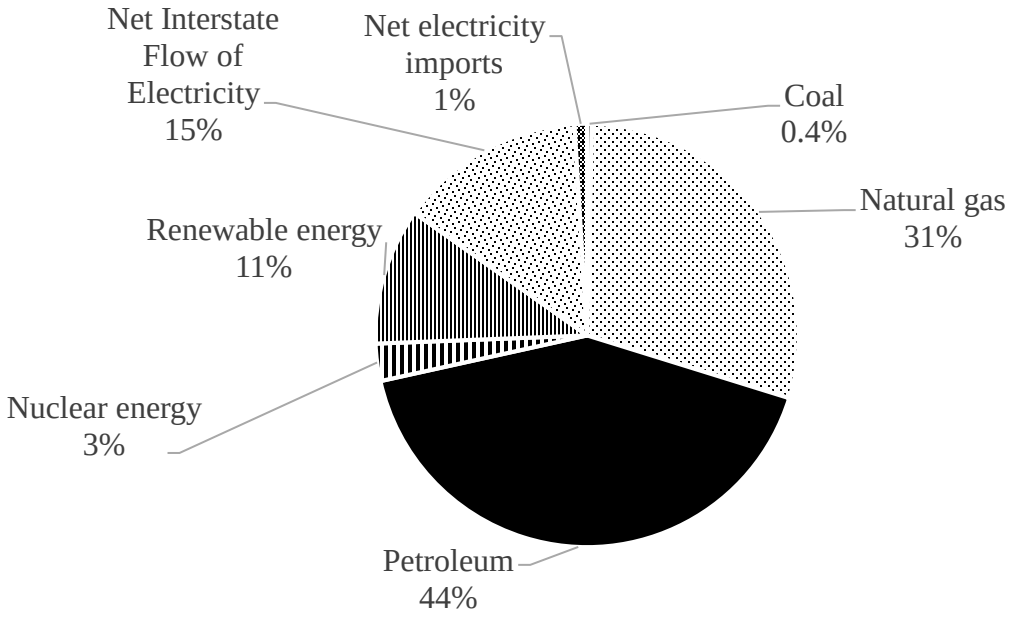

Fig 1.1. Energy consumption for California in 2015 (adapted from EIA, 2017)

One hurdle to overcome is the high insoluble fiber content of olive cake. Olive cake is composed of 12.9 – 16.8%, 10.6 – 18.9%, 12.1 – 30.8% hemicellulose, cellulose, and lignin, respectively, requiring pretreatments convert this insoluble fiber into soluble fiber (Chiofalo et al., 2004, Ferrer et al., 2018).

A biological pretreatment consists of the use of an organism or its enzymes to convert the insoluble fiber in the feedstock into digestible soluble fiber (Sindhu et al., 2016). There are cases where biological pretreatments are used to detoxify certain feedstocks so they can be used for biofuel production (Fountoulakis et al., 2002). Biological pretreatments are considered more sustainable compared to chemical or thermal pretreatments as they do not require complicated, energy-hungry machinery or

dangerous reagents synthesized from petroleum. A disadvantage of using organisms to pretreatment a substrate is the depletion of nutrients necessary to sustain the organism and therefore the treatment. Since these organisms grow on the feedstock (i.e. wood chips) it is pretreating, they will inevitably deplete the substrate of organic, digestible matter. For that reason, biological treatments are considered somewhat ineffective (Zheng et al., 2009). One type of biological pretreatment is enzymatic pretreatment where only enzymes are applied to the substrate. Solely applying enzymes not only produces no waste products, but also the use of enzymes leads to a significantly lower lag-phase which can translate a higher rate of substrate conversion. Furthermore, unlike using an organism to pretreat a feedstock, none of the macromolecules are converted into biomass.

1.2 Statement of research question

Can olive cake be sustainably pretreated to improve the amount of biofuel produced from fermentation with *Sacchromyces cerevisiae* and anaerobic digestion. Can the residue from the anaerobic digestion of olive cake be further used for animal feed?

1.3 Approaches

The first objective of this project was to determine whether destoning by horizontal screw press (HSP) equipped with a screen or destoning by centrifugation removed a larger percentage of stones from the olive cake. It was hypothesized that screening would be more effective at separating the digestible solids and the stones than centrifugal force. To test this hypothesis, enzymatically pretreated olive cake was submitted to destoning by either horizontal screw press (500 rpm) equipped with a 0.71 mm mesh screen or centrifuge (100, 200, 3,000 x g) and the percentage of stones removed and the total solids retained for both processes were compared.

The next objective was to compare the biomethane and bioethanol potential of sustainably pretreated olive cakes (destoned, enzymatically pretreated, or both) and untreated olive cake. Destoning was expected to remove a majority of the indigestible stones, increasing the amount of digestible pulp available to the anaerobic microorganisms or yeast, resulting in higher biofuel yields. The addition of an enzyme cocktail containing hemicellulase, cellulase, pectinase, and xylanase was hypothesized to result in the hydrolysis of cellulose, hemicellulose, and pectin, increasing the concentration of simple carbohydrates available to the microorganisms. To test these hypotheses a control (DOC, untreated olive cake) was compared to destoned (DDOC), enzymatically pretreated (EDOC), and destoned and enzymatically pretreated (DEDOC) olive cakes. To compare the biomethane potential of these four substrates a biochemical methane potential (BMP) was performed, and to compare the bioethanol potential of these four substrates, a batch fermentation with *Saccharomyces cerevisiae* was performed.

Finally, the last objective was to determine if the residue from anaerobically digesting olive cake, digestate, had potential as animal feed. It was hypothesized that the anaerobic digestate would be a good addition to animal feed since digestates are known to have high concentrations of available nitrogen. To test this hypothesis, proximate analysis was performed on the digestate of the pretreated olive cakes that produced the highest amounts of biomethane.

(This research was done as part of a larger collaborative study focusing on different uses for olive mill wastes. Dr. Li from Cal Poly Pomona worked on using OMW as a food ingredient, Dr. de Moura from UC Davis worked on the fungal growth and the

fermentation of olive pomace, and we had help from Dr. Edwards from Cal Poly San Luis Obispo to determine the left-over residue from anaerobic digestion, the digestates', value as animal feed)

CHAPTER 2 – LITERATURE REVIEW

This section will contain a summary and analysis of research related to the pretreatment and valorization of olive mill wastes (OMW). Background information will include the ways olive oil is produced, why the effluents from olive mills are an environmental hazard, and a description of compounds of interest in olive mill wastes.

2.1 Olive oil production

Olive oil production can be separated into two stages: the pressing of olives to make a mash and the extraction of the oil. The first stage consists of crushing the olives to produce a paste. The violent crushing of olives causes emulsions to occur in the olive paste, which can reduce the oil yield. To counteract these emulsions, the olive paste undergoes a gentle heating and stirring process known as malaxation to promote the coalescence of oil droplets. Increasing the length of malaxation significantly increases the amount of oil produced, but results in the production of a lower quality olive oil due to the degradation of phenolic compounds (Di Giovacchino et al., 2002).

After malaxation, the oil is extracted from the paste. There are currently two types of olive oil extraction systems industrially used: the three-phase centrifuge (3PC) and the two-phase centrifuge (2PC) (Vossen, 2007). The olive mill waste used in our study came from a 3PC and therefore the subsequent sections will focus more heavily on 3PC and its effluent streams.

The 3PC was introduced to the olive industry in the 1960s and is still widely used in many Mediterranean olive oil producing countries like Italy, Greece, and Cyprus (Roig et al., 2006). A three-phase centrifuge is a horizontal centrifuge that separates the olive oil from the olive paste by leveraging the weight difference between the oil and the

solids. To do so, the centrifuge requires the addition of 0.6-1.3 m^3 of hot water for every ton of olives (Fig. 2.1) (Azbar et al., 2004). This separates the paste into three fractions: the olive oil, a solid residue known as olive cake or 3POMW, and olive mill wastewater (OMWW). The olive cake and olive mill wastewater constitute the effluent streams that have minimum value.

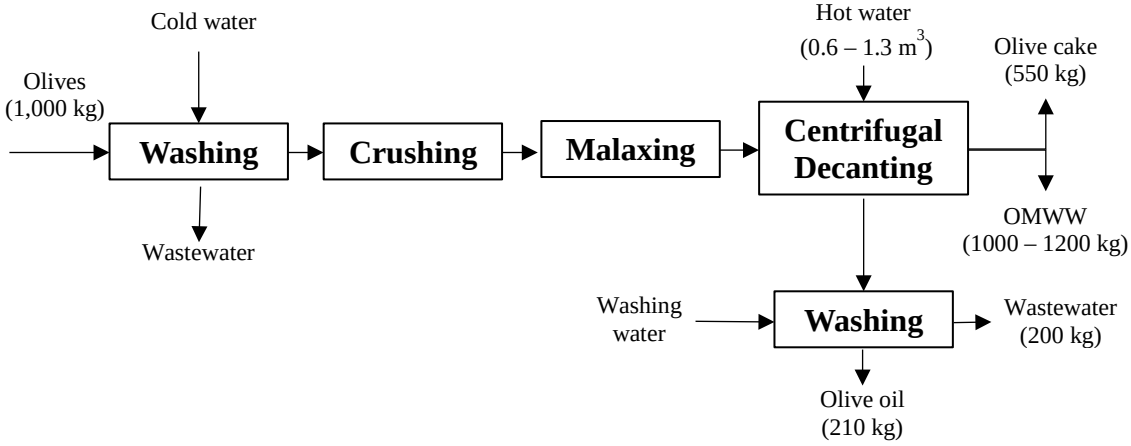

Fig. 2.1. Three-phase olive oil extraction system flowchart (adapted from Vlyssides et al., 1998; Azbar et al., 2004)
OMWW = olive mill waste water

The greatest advantage of a 3PC system over the traditional system that used pressure mats to extract the oil is the reduced need for labor. A 3PP system is continuous and can process three times as many olives in a day. This reduced labor and increased efficiency come with a price as the 3PC system uses 50% more water than the traditional system (Niaounakis and Halvadakis, 2006a). The amount of wastewater (OMWW) generated by the 3PC (1,000-1,200 kg/ton of olives) is greater than the traditional method by a factor of 2 (600 kg/ton of olives) (Azbar et al., 2004). This large amount of wastewater is correlated to a slight reduction in the quality of olive oil produced. The

main antioxidants in olive oil, the phenolic compounds, are mostly hydrophilic molecules and migrate into the aqueous fraction, leaving less in the oil (Borja et al., 2006).

The stones of the olive fruit are left intact when the olives are crushed. The stone fragments contribute to friction and cut pulp cells, increasing the ease of oil release. Removing the stones before crushing can reduce oil yield by 30%, and it has been shown that the enzymes in olive stones greatly contribute to the olive oil aroma during oil extraction (Luaces et al., 2003, Amirante et al., 2006). There is anecdotal evidence from the olive oil industry that removing the stones before crushing the olives increases the quality of the oil. The current body of literature on the topic suggests that destoning olives before crushing makes little or no difference in the quality of olive oil (Patumi et al., 2003; Servili et al., 2007). This may change as there is more interest in destoning technology and destoned olive oil (Restuccia et al., 2018).

2.2 Olive cake composition

The seasonality and locality of olive oil production introduces high variability in the physicochemical properties of olive cake. Factors such as soil quality, rainfall, the olive cultivar, and processing methods, all affect the composition of olive cake (Table 2.1). Olive cake is known to have a **moisture content** of 40-60% and over 90% of the dry matter is organic matter. The majority of the organic matter is made up of cell wall constituents: hemicellulose, cellulose, and lignin (Felizón et al., 2000). The average amount of **lignin** in olive cake (10-30%, db) is similar to the lignin content of straws (10-20%, db). The lignin content in olive cake is often overestimated due to the formation of polysaccharide-protein-phenolic complexes that closely resemble lignin (Chiofalo et al.,

2004, Sánchez, 2009, Ferrer et al., 2018). These complexes form during both fruit maturation as well as when the olives are crushed (Coimbra, 1994).

Table 2.1. Comparison of olive cake composition from different sources

	Olive cake (as is)[a]	Dried olive cake[b]	Dried olive cake[c]	Milled olive cake[d]
Total Solids (g/kg)	498.0 ± 19	919	908	945 ± 5.0
Fats and oils (%TS)	7.8 ± 3.0	1.2	22.1	3.3 ± 0.2
Proteins (%TS)	6.8 ± 0.0	10.2	3.3	9.8 ± 1.3
Hemicellulose (%TS)	15.9 ± 0.8	15.2	8.3	14.6 ± 4.8
Cellulose (%TS)	34.9 ± 0.4	12.8	12.0	27.9 ± 1.5
Lignin (%TS)	20.5 ± 0.4	36.7	6.9	16.8 ± 1.4
Volatile Solids (%TS)	96.6 ± 0.1	90.2	n.d.	92.3 ± 0.6
Ash (%TS)	3.4 ± 0.1	9.8	n.d.	7.7 ± 0.6
Total Phenolics (mg GAE/g TS)	3.4 ± 0.0	n.d.	n.d.	1.2 ± 0.0

All olive cakes are from a 3PC; n.d. = not determined; TS = total solids; GAE = gallic acid equivalent; (a) Vlyssides et al., (1998); (b) Tufarelli et al., 2013; (c) Chiofalo et al., 2004; (d) Felizón et al., 2000, defatted and destoned olive cake has been subjected to vibratory ball milling

The structure of lignocellulosic biomasses such as straw, bamboo, and corn stover consists of cellulosic microfibrils bound by lignin, which acts as a cement. This lignin hinders enzymes from reaching and degrading the cellulose and hemicellulose (Fu et al., 2012).

As olives belong to the drupe category, which includes peaches, cherries, apricots, etc, the olive pulp consists of cellulose microfibrils existing within a matrix of non-cellulosic polysaccharides, oligosaccharides, glycoproteins and phenols held together with a combination of covalent and non-covalent bonds such as hydrogen bonds (Coimbra, 1994). Unlike an agricultural residue like corn stover, most of the lignin in olives is in the stone and the pulp cells that are close to the stone (Coimbra et al., 1995).

The processing conditions have a large effect on the amount of **residual oil** left in olive cake with the amount of oil remaining ranging from 7.8-22.0% (db) (Vlyssides et

al., 1998; Chiofalo et al., 2004). This residual oil is then extracted with the use of solvents such as hexane in a process similar to how seed oils (canola, soybean, sunflower, peanut, etc) are extracted. Solvent extraction can decrease the fat content in the defatted olive cake to 1-3% (db) (Table 2.1). Studies have shown that defatting the olive cake in this manner increases its shelf-life from 4 days to over a year under unspecified conditions (Sansoucy, 1985).

Olives have a low amount of **protein** (3.3-10.3%, db) and are limited in histidine and methionine (Lazovic et al., 1997; Chiofalo et al., 2004; Tufarelli et al. 2013). The extraction of proteins from olives is difficult due to the number of interfering compounds such as polysaccharide-protein-phenolic complexes. In addition, these complexes reduce the bioavailability of olive cake protein when used as an animal feed (Molina Montealegre et al., 2014).

The olive **stones or pits** refer to the kernel and the seed of the olive. The olive stone consists of 25% of the olive's total weight and contains 20-30% w/w of oil. Olive seed oil has 7% less oleic acid than the oil from the pulp but more than two times the amount of linoleic acid, an essential polyunsaturated fatty acid. Stones are removed after olive oil extraction, i.e. from the cake, using cyclones that separate the lighter pulp with the heavier stone fragments for the production of olive pomace oil (Moral and Méndez, 2006). Large and costly, these machines represent an additional financial investment that can be a pitfall for small olive producers. These stone fragments are most commonly burned to recover energy (Hernández et al., 2014). A more sustainable alternative is their use for carbon sequestration or heavy metal sorption (Abdelhadi et al., 2017).

2.3 Environmental impact of olive mill waste

The detrimental effects of olive mill wastes (OMW) on the environment have been reported since ancient Greece and Rome (Niaounakis and Halvadakis, 2006a). When OMW is discharged into the environment, waterways and produces offensive odors. Further research has revealed phytotoxic effects, soil quality loss, increased oxygen demand in waterways, and decreased air quality (Paredes et al., 1987; DellaGreca et al., 2001; Rana et al., 2003).

2.3.1 Land

Olive cake contains a high amount of organic matter, which can be a growth medium for plants and bacteria when used to amend soil. However, the residual oil in olive cake decreases capillary rise and unsaturated hydraulic conductivity in solids. When olive cake is applied to land, plants need more water and it takes longer for the water to reach its roots (Abu-Zreig and Al-Widyan, 2002). For that reason, after all the residual olive has been extracted, olive cake is commonly burned or landfilled (Goldsmith et al., 2018).

An observed phytotoxic effect is observed when olive mill wastewater (OMWW) is applied to soil as a water source or fertilizer. The cause is believed to be OMWW's low pH, its high mineral salt content, and the high concentration of polyphenol (0.5-24 mg/mL) (Paredes et al., 1999; Niaounakis and Halvadakis, 2006b).

Olive mill wastewater has an acidic character (pH 5) and when stored in lagoons or tanks, the fatty acids present in the wastewater are hydrolyzed to form organic acids which further depress the pH. Under this hypothesis it is expected that the heavy application of OMWW would greatly depress the soil pH, rendering the soil unfit for

agriculture. On the contrary, a single, high application of OMWW (160 m^3/ha) has a minimal (0.03 units of pH) effect on the pH of the soil (Marsilio et al., 1990). It has not been tested if continuous applications of a high amount of OMWW significantly affect the long-term acidity of soil. High concentrations of salt have exhibited detrimental effects on plant growth and viability (Parida and Das, 2005). Excessive doses of OMWW (200 m^3/ha) temporarily raise the salinity in unspecified soils (0.36% compared to 0.24% for control soil) (Niaounakis and Halvadakis, 2006b). The polyphenols in OMWW and olive cake have commonly been attributed to its phytotoxicity. In particular, catechol, 4-methylcatechol, tyrosol, and hydroxytyrosol, have shown considerable phytotoxic effects on tomato seeds (Capasso et al., 1995).

Based on OMWW's effect on the pH, salinity, and phenolic content of soils, it was recommended that if applications did not exceed 30 m^3/ha year, using OMWW as soil amendment was beneficial for growing olive trees (Niaounakis and Halvadakis, 2006b). Furthermore, continuous, evenly spread, moderate application of untreated OMWW (50 – 70 m^3/ha year) for seven years had little effect on soil health (Litaor and Khadya, 2018). Such a moderate level of spreading is not feasible for large olive mills that produce large volumes (>1000 m^3/ha) of OMWW.

2.3.2 Waterways

Olive mill wastes used to be discharged into rivers or streams, which caused many of the rivers in the largest olive oil producing countries to become anoxic, destroying native flora and fauna (Niaounakis and Halvadakis, 2006b). In turn, many Mediterranean countries prohibited OMW disposal in rivers. Nevertheless, these residues are still disposed in sewers and the ocean.

The effects of OMWs on municipal wastewater treatment infrastructure are severe. For instance, the high volume of volatile fatty acids in OMWW are known to corrode sewer pipes, causing severe structural damage. Due to its high organic loading, 1 m^3 of OMWW is equivalent to 100-200 m^3 of sewage, which has the potential to overload the local wastewater treatment facility. Wastewater treatment plants close to olive mills have the same design problems as plants in college towns where the student population disappears for the summer. This highly concentrated waste goes through anaerobic digestion in open basins, further increasing the acidity, producing foul odors, and emitting uncaptured methane that contribute to GHG emissions (Rozzi and Malpei, 1996).

2.3.3 Air quality

One of the main drawbacks when treating OMW are the odors produced. In Europe, when olive mills were enjoined from disposing OMW into rivers, olive oil producers dug storage ponds to dispose of their waste. The OMW, which can be stored in an open-air pond for several months, ferments and can produce both methane, a GHG 28-36 times stronger than carbon dioxide, and hydrogen sulfide, a foul-smelling air pollutant (Niaounakis and Halvadakis, 2006b; EPA., 2017). In addition, short chained organic acids that are particularly malodorous like acetic acid, isobutyric acid, and valeric acid are present in the gas due to uncontrolled anaerobic fermentation, hydrolyzing the fatty acids into more volatile compounds (Papaioannou, 1988). These malodors are particularly an issue for olive pomace oil extraction facilities as the extremely pungent odors from the fermenting olive cake are released into the atmosphere as waste gases during the drying process.

Another area of concern is the volatilization of phenol, a genotoxin, when applying OMWW to soil. Up to 650 kg of airborne phenols were emitted from the moderate application of OMWW (50 m^3/ha year) (Rana et al., 2003). The concentration of airborne phenol reached up to 7 µg/m^3, which leads to a real risk of exceeding the total acceptable daily intake for humans, 100 µg/kg male body weight per day (IPCS INCHEM., 2002). Furthermore, the presence of volatilized sulfur dioxide (33 g/ha) is also of concern as it is a precursor for acid rain (Rana et al., 2003).

2.4 Valorization of olive mill waste (OMW)

Until the 1990s, industries solely sought to detoxify or remove pollutants so their waste would be suitable for disposal in a landfill. Due to the rapid depletion of natural resources, a new principle where wastes are converted into valuable materials and energy called valorization emerged (ElMekawy et al., 2014).

2.4.1 Concept of biorefinery

A biorefinery is one model of vertically integrated valorization. Based off petroleum refineries, biorefineries use biomass rather than crude oil as a carbon source. At each stage of the biorefinery, different components of the waste are further processed or extracted to obtain products such as antioxidants or soluble polysaccharides. These high-value products are then sold for profit. After the extraction of more valuable components, the residual biomass is converted into biofuel or incinerated for energy recovery (de Jong and Jungmeier, 2015). The process of retrieving these products requires inputs such as water, biological materials, energy, strong acids, alkalis or even metallic catalysts. On many occasions, this can create more environmentally harmful

waste products (Romero-García et al., 2014). The following section will go over the products produced from the valorization of OMW.

2.4.2. Antioxidant recovery

Water soluble phenolic compounds with antioxidant properties are considered one of the more valuable product that can be obtained from OMW. These phenolics have been shown to have protective effects on human health including but not limited to protection from oxidative DNA damage, low-density lipoprotein oxidation, and inflammation (Cicerale et al., 2008). When olive oil is extracted with a three-phase centrifuge (3PC), 2% of the phenolic compounds are found in the oil, 53% are partitioned into the wastewater, and 45% is retained in the olive cake. The most abundant and important phenolic compound in OMW is hydroxytyrosol (0.8% of dry olive cake) which is the degradation product of oleuropein, a glucoside that contributes to bitterness and is the most abundant polyphenol in olives (Fig. 2.2) (Lesage-Meessen et al., 2001; Rodis et al., 2002).

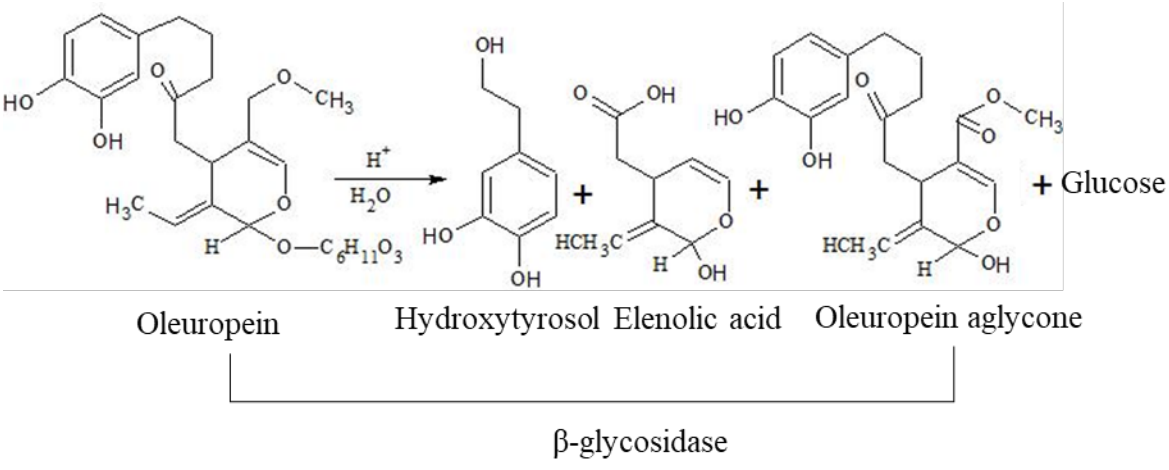

Fig 2.2. Oleuropein and its hydrolysis products (adapted from Niaounkais and Halvadakis., 2006a)

Phenolic compounds in dried olive cake can be extracted with a 29% yield using ethyl acetate (Lesage-Meessen et al., 2001). This low yield is due to ethyl acetate's selectivity for small and medium molecular weight phenolic compounds. The other solvent commonly used to extract phenolic compounds from OMW is methanol. The extraction of phenolic compounds from two-phase olive mill waste (2POMW), the semi-solid effluent produced from a 2PC, with 60% v/v methanol resulted in the recovery of three times as many polyphenols as ethyl acetate extraction (Obied et al., 2005).

The storage method of the OMW also impacts the amount of phenolic compounds extracted. The highest yields of phenolic compounds are obtained when OMW is freeze dried, but lyophilization uses excessively high amounts of energy. Therefore, it is recommended to dry OMW at low temperatures to reduce the polyphenol degradation.

Methanol also extracts some of the residual oil in olive cake, so the resulting extract would need to be defatted with hexane, a reagent produced from petroleum, to segregate the oil, before the extract can be used (Obied et al., 2005).

Other extraction methods involve alkaline conditioning before organic solvent extraction and solid-liquid extraction at high pressure. In all cases, high amounts of energy are necessary and even more waste products are created (Suárez et al., 2009).

Sustainable and affordable phenolic extraction serves as the cornerstone to a profitable OMW biorefinery, but with current extraction methods, the environmental disadvantages are obvious. There have been advances in this field as there is great interest in sustainably recovering phenolic compounds. The most promising nonconventional extraction methods are the use of natural deep eutectic solvents (NADES) and supercritical fluid extraction (SFE).

Natural deep eutectic solvents (NADES) are solvents that are composed of metabolites that are naturally present in organic matter. The common components of these solvents are simple carbohydrates (glucose, fructose, etc), organic acids (lactic, malic, etc), urea, and choline chloride. Ultrasound (30 min, 200 W output power) was used in tandem with LGH (lactic acid and glucose, 5:1) to extract phenolic compounds from two-phase olive mill waste. About 95% of the total hydroxytryosol was extracted, which was only 2-3% less than the amount of hydroxytyrosol extracted with methanol (Fernández et al., 2018).

Supercritical fluid extraction (SFE) is another alternative phenolic extraction method. The solvent most commonly used is carbon dioxide due to its selectivity in extracting phenolic compounds with minimal environmental impact (Le Floch et al., 1998). Extraction with supercritical carbon dioxide at 40 °C and 35 MPa for 60 min was able to yield 7.6 mg caffeic acid equivalent/g total solids (TS) from 2POMW, which was almost 60% of the total phenols extracted with pure methanol (Lafka et al., 2011). One pitfall of supercritical fluid technology is the energy necessary to pressurize CO_2 so it will turn into a supercritical fluid.

2.4.3 Pectin recovery

Citrus peel or apple pomace are the only available sources of commercial pectin. They are highly susceptible to molding due to their carbohydrate-rich composition and high moisture content (Cardoso et al., 2003). The molds produce a variety of pectinases that affect the quality of the final pectin. Therefore, there is always the need for alternative pectin sources (May, 1990).

Pectic polysaccharides make up a third of olive pulp cell walls. These pectins are known as “arabinans” because they are mainly composed of α-(1→5)-linked L-arabinofuranose units. Due to this composition, arabinans have a higher methyl esterification than the commercial low-methoxyl pectins (43% vs 35%, respectively) and a higher degree of acetylation (11% vs >1%, respectively) (Coimbra et al., 2010). A higher degree of methyl esterification increases gelling capacity, while a higher degree of acetylation inhibits gelling (Melton and Smith, 2001). Furthermore, olive pectins contain eight times the concentration of higher neutral sugar side chains. These side chains hinder interaction between pectin chains, limiting access to calcium, the primary gelling catalyst. Therefore, OMW pectins require a higher concentration of calcium to achieve the same gel strength as commercial pectins (Cardoso et al., 2003). But the abundance of neutral sugar chains means that they are more stable at high temperatures (> 50 °C) than commercial low-methoxyl pectin gels due to the abundance of neutral sugar chains. These neutral sugars form significant non-ionic interactions (hydrogen bonds, hydrophobic interactions), which is an phenomenon observed in high-methoxyl pectins (Coimbra et al., 2010). It was concluded that olive pectins from OMW are a promising alternative natural source for commercial pectins due to their gelling ability and temperature resistance.

2.4.4 Compost

The excessive use of untreated OMWW as a fertilizer (> 90 m^3 ha/ year) is prohibited by law in some Mediterranean countries because extreme application of OMW exhibit phytotoxic effect or are detrimental to the soil quality (Section 2.3.1). One method

of circumventing this regulation and the phytotoxic properties of untreated OMW is composting OMW.

Composting refers to the partial aerobic degradation of the organic fraction of a biomass into carbon dioxide, water, and a fertilizer (Baeta-Hall et al., 2005). Composting can be divided into three stages: the preprocessing, the active composting, and the stabilization. The preprocessing of OMW is rather extensive. Other feedstocks usually need to be added to increase the porosity of OMW, dilute the concentration of phenolic compounds, and increase the nitrogen content. In some cases, urea must be added to ensure the optimal C/N ratio, ~30 (Tomati et al., 1995). During the active (thermophilic) stage of composting, which can last from a few days to several months, fungi that produce lignin degrading enzymes (laccase, lignin peroxidases) develop in the pile. This same enzyme is also capable of breaking down phenolic compounds. In one case, a lignin and polyphenol reduction of 70% was seen after the thermophilic phase (2 weeks) (Galli et al., 1997). The stabilization phase is responsible for the production of humic acids and fulvic acids. The higher amount of these substances means a higher quality organic fertilizer.

To test the feasibility of composting OMW, Vlyssides et al. (1996) a demonstration facility, which co-composted olive cake and OMWW was designed and constructed. The demonstration facility was able to treat 22% of the OMWW (263 m^3) produced by a nearby olive mill in 23 days of operation followed by a 3-month stabilization period. Olive cake (0.3 kg olive cake/kg of OMWW) was used as a bulking agent.

Although co-composting OMW may alleviate a fraction of the growing amount being produced, it cannot be the sole solution for the valorization of OMW. For example, California has very stringent quality standards for finished compost (CalRecycle, 2004). When Michailides et al. (2011) co-composted olive leaves and olive cake, the highest germination index was obtained (198%), a measure of germination speed, reported for compost produced with olive cake. Yet, this compost would not pass California quality standards and therefore could not be marketable in California (Table 2.2). Since there is a low economic incentive for the production of compost and quality standards in California are difficult to achieve when composting olive cake alternative uses for olive cake must be considered.

Table 2.2. Comparison of physicochemical properties of olive cake compost with California quality standards (adapted from CalRecycle, 2004; Michailides et al., 2011)

	CalRecycle Standards	Michailides et al., 2011
C/N ratio	<17:1	27.1
Organic Matter (%)	20 - 35	37.4
Total Nitrogen (%)	1.0 - 2.0	1.79
Nitrate (ppm)	0	n.d.
Nitrite (ppm)	0	n.d.
Sulfide (ppm)	0	n.d.
Ammonium (ppm)	0 or trace	n.d.
pH	6.5 - 8.5	7.58
CEC (meq/100g)	>60 meq/100g	n.d.
Humic Acid Content (%)	5 - 15	n.d.

Standard deviations were not reported. n.d. = not determined

2.4.5 Animal feed

Crude olive cake has limited application as an animal feed. It is composed of a high amount of insoluble fiber (60% neutral detergent fiber, NDF) and a low amount of protein (5.5%, db). These properties make it difficult to digest for ruminants with only 32% of the dry matter being digested after 72 h in the rumen (Sansoucy, 1985).

When alkali is added to olive cake, lignin is broken down, increasing the availability of cellulose. The alkali also disrupts the bonds forming the polysaccharide-protein-phenolic complexes, increasing the availability of protein. When 2% w/w of ammonia was added to ensiled olive cake, the crude protein available to the ruminant increased by 40%. However, when alkali is added to crude olive cake, the residual oil is saponified, forming inedible soap. This reaction can be overcome by first extracting the residual oil from olive cake before treating it with ammonia or using an alkali that does not cause a saponification reaction (NH_4OH, Na_2CO_3) (Sansoucy, 1985). Industrially, ammonia, an organic fertilizer, is produced using the Haber-Bosch, process which requires high amounts of heat (400-500 °C) and natural gas. The unsustainable use of artificial fertilizers derived from fossil fuels is a major concern for Californian agriculture. Large scale alkali pretreatment of olive cake for animal feed may not be environmentally nor economically viable (Wood and Cowie, 2004).

One benefit to feeding animals crude olive cake is the presence of oleic acid in the olive cake. Oleic acid makes up a majority of the residual oil in olive cake. Feeding crude olive cake to ewes significantly increased the oleic acid content of ewe's milk by 33%, while decreasing the saturated fatty acid content (Chiofalo et al., 2004). Animal products with a lower ratio of saturated fatty acids to unsaturated fatty acids are known to be heathier for humans (Scollan et al., 2001).

2.4.6 Energy

Olive cake has a caloric value of 12.5 to 21 kJ/g TS, which is comparable to wood (17 kJ/g TS) and soft coal (23 kJ/g TS). Historically, olive cake was combusted in rural Mediterranean regions as an alternative fuel for coal. Therefore, the generation of

electricity or heat from the combustion of olive cake is already a well-established industry in the olive oil producing areas of Europe (Berbel and Posadillo, 2018).

During the combustion of olive cake, carbon monoxide, hydrocarbons, and fine particles form. All of these products are environmentally hazardous when expelled into the atmosphere, and the fine particles easily clog filters, increasing the frequency filters need to be replaced (Atimtay and Topal, 2004; Moral and Méndez, 2006).

Fluidized bed combustion is used to burn solid fuels on a bed of solid particles, usually sand, that has been preheated with pressurized gas until it behaves as a liquid. When the solid fuel is fed into the preheated sand, it combusts, heating water which then can be converted into steam and used to generate electricity. Fluidized bed combustion has been used to incinerate a wide range of agricultural materials including olive cake. In all cases, the energy yield from the combustion of olive cake was lower than that of coal. The advantage of combusting or co-combusting olive cake is that it has a much lower SOx emission (0-35 mg/N m^3) compared to coal (3,000 mg/N m^3) (Topal, 2003). As a biomass, olive cake has a high ash content (8.3%) and therefore high turnover of dust collectors and a rigorous post-treatment of exhaust gas is necessary, increasing the cost of this technology (Niaounakis and Halvadakis, 2006c; Ferrer et al., 2018).

2.4.7 Fuel

The fuels that can be made from OMWs are biodiesel from triglyceride transesterification, bioethanol from fermentation, biomethane from anaerobic digestion, and gasification or pyrolysis products. Biodiesel is currently not a popular choice as residual oil from olive cake can be processed into olive pomace oil, which has a higher value (Che et al., 2012).

2.4.7.1 Ethanol production

Bioethanol production from olive cake can be separated into three categories: the use of modified or unconventional organisms, the use of pretreatments to increase the carbohydrate availability, and a combination of the two.

When olive cake (5% total solids (TS)) was fermented with *Thermoanaerobacter* ethanolicus, 0.65 mg/mL of ethanol was produced. This was 62% of the control (glucose media). When the total solids loading was increased by a factor of 2, the amount of ethanol produced decreased by 30%. This reduction in the amount of ethanol produced was attributed to potential unspecified inhibitors in the olive cake (Jurado et al., 2010).

A combination of commercial enzymes that contained cellulase, hemicellulase, and beta-glucosidase activities (Celluclast 1.5L and Novoenzyme 188) and a wet oxidation pretreatment (1.2 MPa oxygen at 185 °C) was used to pretreat 2POMW before fermentation with *Saccharomyces cerevisiae*. The highest amount of ethanol (3.24 mg/mL) produced was with the enzyme cocktail but without the wet oxidation. Ethanol production of 2POMW that had undergone wet oxidation as well as enzymatic pretreatment produced 65% less ethanol due to the formation of inhibitory products during the wet oxidation (Haagensen et al., 2009).

An example where olive cake has been pretreated and then an unconventional microorganism has been used for fermentation is when olive cake was pretreated with sulfuric acid at 160 °C and then fermented with *E. coli* FBR5. *Escherichia coli* FBR5 was chosen because it is not only able to convert hexoses but it can also convert pentoses such as xylose into ethanol. Xylose is the monomeric form of xylans which make up 21% (db) of olive cake. Pretreating the olive cake with sulfuric acid hydrolyzes the xylans into

digestible xyloses. When the olive cake was pretreated with 1.75% w/v sulfuric acid at 160 °C, 8.1 mg/mL of ethanol, 88% the theoretical yield, was produced (El Asli and Qatibi, 2009).

Olive mill wastewater has been used as a growth medium for bioethanol production using yeast, in particular, *Saccharomyces cerevisiae*. Fermentation of *Saccharomyces cerevisiae* p-3 produced 0.5 mg/mL of ethanol in 15 days. Replacing *S. cerevisiae* with *Torulopsis sp.* MK-1 to ferment OMWW produced significantly more ethanol (12.8 mg/mL) as *Torulopsis sp* are able to utilize xylose (Yablochkova et al., 2003). Ethanol yields did not come close to the amount of ethanol produced when grape juice was fermented with *Saccharomyces cerevisiae* p-3 (95 mg/mL) (Bambalov et al., 1989). The phenolic compounds in OMW are known to inhibit *Saccharomyces cerevisiae* when concentrations are higher than >3.8 mg gallic acid equivalent (GAE)/mL. When 55% of the phenols were removed from OMWW and then fermented with *Saccharomyces cerevisiae*, the same ethanol yield was obtained as when a mixture of OMWW:water (55:45) was fermented. Both treatments were supplemented to have an equal amount of reducing sugars when the fermentation began. (Zanichelli et al., 2007).

Harsh pretreatments such as high pressure, high temperature and strong acid of olive cake can result in the production of unforeseen inhibitory products, which can then result in decreased ethanol yields. Equally problematic for the fermentation of OMWW is the high concentration of phenolic compounds. Even if all these hurdles are overcome, the amount of ethanol produced is paltry compared to current feedstocks like corn (169 mg/mL) (Lee et al., 2016).

2.4.7.2 Biogas production

Anaerobic digestion takes advantage of the high organic fraction (volatile solids) of OMW to produce biogas, which is primarily composed of CO_2 and CH_4. Low concentrations of hydrogen sulfide can also be present in biogas if substrates high in protein, especially cystine, are digested (Dhar et al., 2011). Anaerobic digestion includes four main sequential steps: hydrolysis, fermentation (acidogenesis), acetogenesis, and methanogenesis (Fig 2.3).

The first and normally rate limiting step in anaerobic digestion is the hydrolysis of complex organic molecules, i.e. carbohydrates, proteins, and lipids into carbohydrate monomers, amino acids, and fatty acids, respectively. Fermentative bacteria then ferment the carbohydrate monomers, amino acids, and fatty acids into intermediary products such as organic acids, alcohols, and ketones. After this step, the process splits into two different paths. One path begins with the fermentation of simple carbohydrates, amino acids, and their intermediary products into acetic acid. Acetic acid is subsequently decarboxylated to produce methane and carbon dioxide in a process called aceticlastic methanogenesis. This pathway is the predominant methanogenic pathway and produces 66-70% of the methane from anaerobic digestion. The second pathway begins with the β-oxidation of fatty acids by acetogenic bacteria to produce hydrogen and carbon dioxide. Hydrogen and carbon dioxide can either undergo homoacetogenesis, the conversion of carbon dioxide and dihydrogen into acetic acid via the acetyl-CoA pathway, by homoacetogenic bacteria, or they can undergo reductive methanogenesis, the reduction of carbon dioxide and dihydrogen to form methane by, hydrogentrophic methanogens (Ye et

al., 2014). The methane produced from the second pathway accounts for 30-33% of the methane produced in anaerobic digestion.

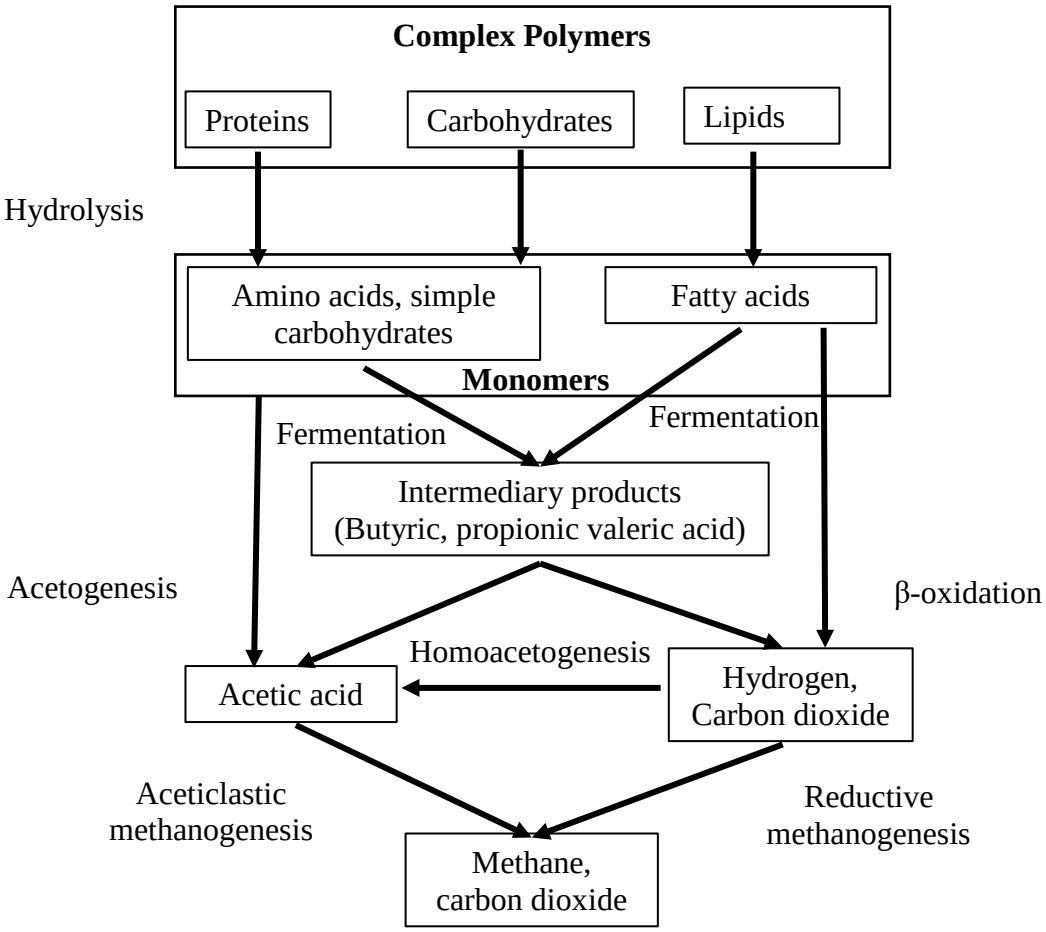

Fig. 2.3. Anaerobic digestion flow chart (adapted from Nayono et al., 2009)

The benefits of anaerobic digestion include the treatment and conversion of a biomass, which would otherwise be landfilled, into an energy rich fuel (upgraded biogas, natural gas) and a mineral and nitrogen rich effluent that can be used as a soil amendment (Nayono et al., 2009; Li et al., 2018).

There are many pitfalls when anaerobically digesting OMW. The presence of phenolic compounds is one reason why a pretreatment is often performed before the digestion of OMW and often the reason why the anaerobic digestion fails to produce an

adequate amount of methane (Hamdi, 1996). High concentrations of lipids can be present in OMW if the residual oil is not extracted. As OMW is high in oleic acid (~70% of total lipid), its lipids are hydrolyzed into long chain fatty acids such as oleic acid during the first stage of anaerobic digestion (Chiofalo et al., 2004). Long chain fatty acids are acutely toxic to anaerobic microorganisms and will inhibit the β-oxidation process as well as methanogenic processes (Hanaki et al., 1981). The overproduction of volatile fatty acids such as acetic acid, an important substrate in aceticlastic methanogenesis, depresses the pH of the digester. The Gibbs free energy necessary for acidogenic conversion is the lowest of all anaerobic conversion, so volatile fatty acids are produced faster than methanogens can convert them into neutral methane. Methanogens only convert acetic acid into methane at a near neutral pH of 6.5-7.5, while acidogens can continue making acetic acid at a pH of 4. Therefore an accumulation of volatile fatty acids eventually leads to acidic conditions, which reduces methanogenic activity (van Lier et al., 2008). This can be countered by having a high amount of bicarbonate alkalinity. The most common source of alkalinity is the calcium carbonate that is already present in the inoculum. If the amount is insufficient, calcium carbonate from an external source must be added.

There is a scarce amount of literature on the anaerobic digestion of olive cake due to well-established disposal of it by co-combustion in the Mediterranean only a few studies can be found. Tekin and Dalgıç (2000) obtained approximately 21 mL CH_4/g volatile solid (VS) from olive cake after a 14 day digestion period using a substrate:inoculum ratio of 2 by VS with a working volume of 1 L. Under the same conditions, the anaerobic digestion of pine produced 59 mL CH_4/g volatile solid (VS) (Chynoweth et al., 1993). When untreated 2POMW was digested under similar

conditions, 213 mL CH_4/g VS was produced (Pellera and Gidarakos, 2016). This discrepancy and the lack of literature justifies the need for further investigation of the biomethane potential of olive cake.

2.4.7.3 Gasification and pyrolysis

Gasification and pyrolysis are thermo-chemical techniques akin to combustion. Pyrolysis is the method of combusting biomass under anoxic conditions to produce a liquid fuel known as “bio-oil” while gasification is the combustion of fuel with limited oxygen to produce “syngas.” Olive cake bio-oil produces about 31.8 kg/g TS, which is higher than what is obtained with most lignocellulosic residues. For example, switchgrass and corn stover produced 23.6 MJ/kg and 24.3 MJ/kg, respectively (Mullen et al., 2009; Şensöz et al., 2006).

Olive cake has been co-gasified with coal to reduce the NOx and SOx emissions due to the low amount of nitrogen and sulfur in olive cake. To avoid destabilizing the reactor, it was recommended to keep the amount of olive cake below 40% w/w (Andre et al., 2005).

Pyrolysis requires high temperature (400-500 °C) and high pressure (0.6 MPa) and gasification requires even higher temperatures (700-800 °C) and the resulting gas needs to be purified before storage or use.

2.5 Olive mill waste pretreatments

Lignin can make up to 30% of the dry matter of olive cake (El Asli and Qatibi, 2009). Pretreatments are used to break down the crystalline structure of the lignocellulosic material. Their common goal is to break the lignin thereby releasing the cellulose and hemicellulose stored within (Fig. 2.4). As most of the lignin in OMW is

concentrated in the stones, most of these pretreatments are aimed at solubilizing the stones. These treatments are categorized based on how the pretreatment is applied to the olive cake, the necessary chemicals, or microorganisms used. Combination pretreatments such as dilute acid steam explosion exist, but they do not belong to a distinct category (Zheng et al., 2009).

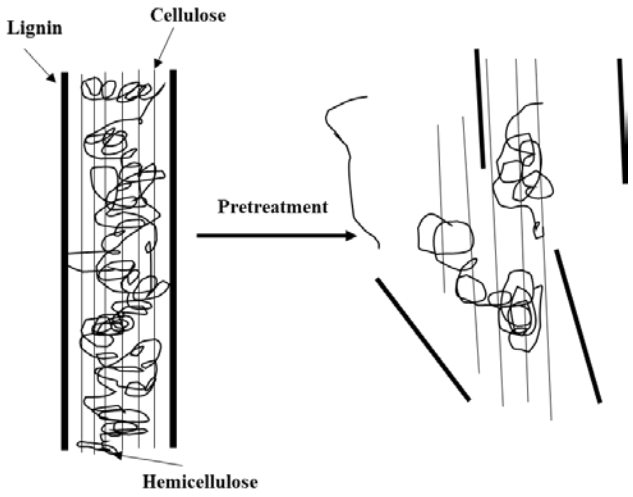

Fig. 2.4. Schematic of pretreatment effect on lignocellulosic biomass (adapted from Mosier, 2005)

2.5.1 Physical pretreatments

Physical pretreatments do not use chemical or biological agents and are usually mechanical or thermal treatments. Thermal treatments are more popular and regarded as more effective but require excessive amounts of energy.

2.5.1.1 Uncatalyzed steam-explosion

Steam-explosion is the most common pretreatment for lignocellulosic biomasses. It is one of the few pretreatments that has gone beyond laboratory-scale experiments to be investigated on an industrial level (Mulat et al., 2018). As the name suggests, steam-explosion involves the high pressure steaming of the biomass lasting from seconds to several minutes to hydrolyze the hemicellulose. This is followed by rapid, explosive

decompression (Romero-García et al., 2014). At high temperatures, water functions as an acid hydrolyzing the acetyl groups of the hemicellulose. These acetyl groups are converted into organic acids, which further promote the hydrolysis of the hemicellulose (Cara et al., 2007). Therefore, the final product has a reduced biomass particle size and increased pore volume, improving enzyme accessibility (Mosier, 2005).

When defatted and destoned olive cake was treated with steam, there was a 64% decrease in glucose as the pretreatment became more severe (higher time and temperature). On the other hand, there was a 450% large increase in xylose, which was expected since most of the hemicellulose in olive cake is composed of xylans (Coimbra et al., 1994). The cellulose in the insoluble fraction was nearly amorphous, which should increase enzyme accessibility. Increasing the severity of the pretreatment had no effect on the concentration of phenolic compounds. It was expected that steam explosion would increase the amounts of phenolic compounds since lignin, which would be degraded, is composed of aromatic compounds. However, there is not much lignin in destoned olive cake since most of lignin is part of the stones. In addition, high temperatures used for the pretreatment (193-234 °C) degrade the phenolic compounds that are already in the pulp (Felizón et al., 2000).

It is possible to combine steam-explosions with acids and bases like sulfuric acid or ammonia to increase the efficiency, but disposal of the waste produced after the pretreatment becomes a concern (Silva et al., 2017). Another disadvantage of steam explosion is the production of levulinic and formic acids from the glucose liberated from cellulose, or furfural and formic acid from hemicellulose due to the high temperatures

used. These products are known to inhibit subsequent enzymatic hydrolysis or bioethanol production (Klinke et al., 2003).

2.5.1.2 Wet oxidation

Wet oxidation is a process similar to steam-explosion. The lignocellulosic biomass is treated with water at pressures ranging from 0.5 to 2 MPa and temperatures higher than 120 °C with oxygen as a catalyst. Like steam-explosion, acid and alkali can be added to increase the effectiveness of the process (Biswas et al., 2015). Alkaline wet oxidation has been used as a pretreatment with the objective of increasing the bioethanol yield of 2POMW. The oxidation was performed in a 2 L vessel pressurized at 1.2 MPa with oxygen. The temperature was 185 °C, 1 gram of Na_2CO_3 was added, and the oxidation lasted for 15 min. The pretreatment decreased the bioethanol yield by 46% due to the production of the same inhibitory compounds mentioned in Section 2.5.1.1. (Haagensen et al., 2009).

2.5.1.3 Destoning

When applied as a pretreatment to olive cake, destoning refers to the separation of the olive stone fragments from the pulp with screens or ventilation. One common method of removing stones after oil extraction but before residual oil extraction is to use a separation machine that blows air against the cake, pulling off lighter pulp particles and leaving behind the larger stones (Intelligent Energy Europe, 2008). Since this process leaves behind a small proportion of the stones, the resulting cake is known as partly destoned olive cake.

2.5.2 Chemical pretreatments

2.5.2.1 Acid pretreatment

The objective of using acid to pretreat biomass is to solubilize the hemicellulose, allowing indigenous enzymes have greater access to the cellulose (Zheng et al., 2009). While sulfuric acid is the most commonly used acid for pretreating olive cake, mineral acids like phosphoric acid have been used on olive tree pruning (Romero-García et al., 2014). The main disadvantages of using acid are material corrosion, the reduction of biomass particle sizes to maximize acid accessibility, and the neutralization required before any other processes are performed. The neutralization is not only especially expensive due to the cost of alkali or washing equipment, but also can have severe impacts to the environment (Zheng et al., 2009; Edeseyi et al., 2015).

When olive cake was pretreated with 1.25% w/v sulfuric acid at 180 °C for 10 min the soluble sugar content was 22% higher than when it was pretreated at 160 °C with the same concentration of acid and for the same amount of time. When fermented with *E.coli* FBR5, the olive cake (pretreated at 180 °C) did not produce any ethanol due to inhibitory products from the pretreatment while the other olive cake (pretreated at 180 °C) produced 85% of its theoretical yield (El Asli and Qatibi, 2009). This result not only highlights the role temperature plays in dilute acid hydrolysis, but also indicates a higher initial concentration of digestible substrate (soluble sugar) does not result in a higher yield of desired product (ethanol).

2.5.2.2 Alkali pretreatment

Traditionally used in the production of table olives, i.e., olives that are consumed, alkali treatments are used to debitter olives by degrading the phenolic compounds

(Sánchez Gómez et al., 2006). Alkalis such as NaOH, NH_3 or $Ca(OH)_2$ (lime) solvate the lignocellulose, causing it to swell, increasing enzyme accessibility. The alkali then saponifies and cleaves the uronic ester linkages cross linking the xylans severing hemicellulose-to-lignin bonds. This disrupts the entire lignocellulosic structure and provokes the destruction of the hydrogen bonds in the cellulose (Rabemanolontsoa and Saka, 2016). The efficiency of the NaOH pretreatment was shown to be dependent on temperature, NaOH concentration, treatment time, and the type of lignocellulose biomass treated (Leite, 2015).

The effect of an alkali pretreatment on the biomethane potential of olive cake has been investigated. Alkaline pretreatment was chosen as it not only could solubilize the lignin (olives stones) but it could also neutralize the volatile fatty acids naturally present in olive cake. Therefore, the pH during anaerobic digestion would not be too acidic. When digested for 50 days at a substrate:inoculum ratio of 0.25 by volatile solids at 35 °C, the olive cake that had been pretreated with 1 mmol NaOH/g VS at 90 °C produced 242 mL CH_4/g VS (Pellera et al., 2016). No control was tested, but this methane yield was more than ten times higher than the result obtained by Tekin and Dalgıç (21 mL CH_4/g VS).

2.5.3 Biological pretreatments

2.5.3.1 Fungi

The benefit of using delignifying fungi to pretreat lignocellulosic biomass is that they excrete natural enzymes, which delignifying the biomass. However, the fungus consumes the biomass as a nutrient source, depleting the biomass of valuable compounds. Anaerobic microorganisms are not included in this category as they lack enzymes such as

lignin peroxidases (lignin peroxidase, manganese peroxidase) and laccases, which digest lignin (Shraddha et al., 2011). Lignin peroxidase is a unique enzyme with a low pH optimum (3), which uses the oxidation of veratryl alcohol as a cofactor to oxidize the nonphenolic parts of the lignin. Manganese peroxidase targets both nonphenolic and phenolic parts of the lignin. It oxidizes Mn (II) into Mn (III) and then oxidizes the phenol rings to phenoxy radicals. This eventually leads to the decomposition of the targeted compounds. Laccase, a copper-containing enzyme, catalyzes the oxidation of phenolic units in lignin as well as other phenolic compounds into radicals using oxygen as an electron acceptor (Binod et al., 2011). All three delignifying enzymes are present in white rot fungi, which has been used to reduce the concentration of phenolic compounds from OMWW (Blanquez et al., 2002).

Wood-rotting fungi are mostly used to reduce the phenolic and lignin content in two-phase olive mill waste (2POMW) to decrease the phytotoxicity of the waste for subsequent composting operations. Lignin removal from 2POMW did not exceed 26% when *Phlebia radiata*, a white-rot fungus, was used to colonize 2POMW for 20 weeks. The fungus was able to remove 95% of the phenolic compound from the 2POMW. This decrease in phenolic compounds had a significant but marginal effect on the phytotoxicity of the residue. When *Lycopersicum esculentum*, tomato plants, were grown in soil amended with *P. radiata* colonized 2POMW, 71% of the growth was inhibited. While this inhibition may be 30% lower than the non-inoculated 2POMW (98% growth inhibition), a 71% growth inhibition was deemed to be unacceptable for industrial use (Sampedro et al., 2007).

The biggest attraction of using fungi to pretreat OMW rather than mechanical and chemical pretreatments are the low energy inputs, negligible chemical requirements, mild environmental conditions, and environmentally friendliness (Sun and Cheng, 2002). But it has also been reported that living organisms are slow to pretreat biomass, requiring weeks when thermal and chemical pretreatments can take minutes or hours. This type of biological pretreatments also requires constant maintenance because the treatment depends on the organism's survival (Chandra et al., 2007). Due to these disadvantages, biological pretreatments using living organisms are considered cumbersome to use on an industrial scale.

2.5.3.2 Enzymatic hydrolysis

The principle behind using enzymes to hydrolyze lignocellulosic biomasses is to eliminate the intermediate organism, the fungi or aerobic microorganism. Enzymes are biological catalysts that can function without nutrients as long as there is enough cofactor in the environment and that the conditions (temperature, pressure, pH) are not too extreme for the specific enzyme. Therefore, enzymatic pretreatment is just as sustainable as pretreatment with a living organism since no hazardous waste is produced. Moreover, neither the growth of an organism nor the nutrients to sustain the organism are necessary (Brummer et al., 2014).

There is scarce data on the enzymatic hydrolysis of olive cake. However, the simultaneous saccharification and fermentation (SSF), a bioethanol production method where enzymes are loaded into a fermentation vessel with yeast to optimize ethanol yield, of 2POMW has been reported (Ballesteros et al., 2002; Haagensen et al., 2009). The objective of the enzymatic hydrolysis of OMW is to convert the insoluble fiber

(hemicellulose, cellulose), which makes up the majority of the pulp, into soluble fiber. In addition, there has been a surge of interest in the enzymatic hydrolysis of agricultural residues like olive cake to increase biofuel production (Saha et al., 2017, de Almeida Antunes Ferraz et al., 2018). The classes of enzymes commonly used to saccharify these residues are cellulase, hemicellulase, and pectinase. Cellulases are a subcategory of glycoside hydrolases and are composed of a mixture of three classes of enzymes that hydrolyze the β-1,4 linkage in cellulose (Sandhu et al., 2018). Endo-β-1,4-glucanases randomly hydrolyzes internal β-1,4-glucosidic linkages increasing the number of cellulose chains. Exo-1,4-β-D-glucan cellobiohydrolases progress along the chain and hydrolyzes the reducing and non-reducing ends of the cellulose polymer. This activity releases glucose and cellobiose, the monomer and disaccharide of cellulose, respectively. Finally, β-glucosidases hydrolyze the cellobiose or cellodextrin in glucose (Fig. 2.5) (Binod et al., 2011).

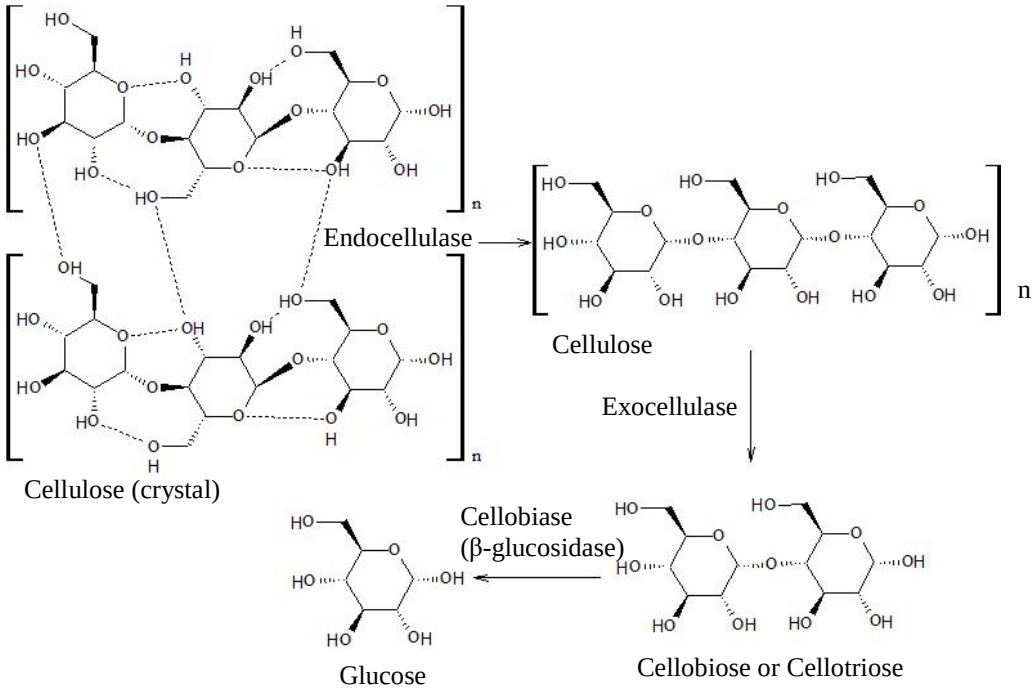

Fig. 2.5. General reactions for cellulases

Exo-1,4-β-D-glucan cellobiohydrolases and endo-β-1,4-glucanases have cellulose-binding domains, which dock the enzyme onto the cellulose to ensure the substrate is in the correct orientation. These binding domains limit the movement of these enzymes to a two-dimensional plane. Furthermore, due to the progressive nature of exo-1,4-β-D-glucan cellobiohydrolase, it can only move laterally across the polymer. This limited movement makes the hydrolysis of lignocellulose, a heterogenous substrate difficult (Bansal et al., 2009). Moreover, it has been reported that the phenolic degradation products of lignin inhibit cellulase activity through an unknown mechanism (Ximenes et al., 2010).

Hemicellulose is a very heterogenous group of branched and linear polysaccharides. Consequently, hemicellulases are a broad group of enzymes that must act in concert to completely degrade hemicellulose. Much like cellulases, hemicellulases have carbohydrate-binding domains that target and bind to specific carbohydrates. Each of the six major classes of hemicellulase either hydrolyzes glucosidic bonds or hydrolyzes ester linkages in acetate or ferulic acid side chains (Fig. 2.6).

β-Mannanase, or specifically, 1,4-β-D-mannan mannohydrolases, hydrolyzes linear mannans and glucomannans. Mannans are a common antinutritive factor present in the cell wall of legumes and their presence is particularly problematic in soybean feed (Jackson et al., 2018). β-Mannanase hydrolyzes glyosidic bonds, releasing short β-1,4-manno-oligosaccharides, which are no longer antinutritive, from the mannan backbone. The oligosaccharides are then hydrolyzed into monomeric mannose by β-mannosidase (van Zyl et al., 2010).

There are two types of arabinases that are considered hemicellulases, α-L-arabinofuranoside, which is exo-acting, and α-L-arabinase, which is endo-acting. These enzymes hydrolyze arabinofuransoyl, which is a common sidechain in hemicellulose that hinders enzymatic access to cellulose and the xylan portion of hemicellulose (Numan and Bhosle, 2006). In olive cake, α-L-arabinases are able to degrade the (1→5)-α-L arabinan chains that make up one-third of the olive pulp cell walls.

Xylan, the main carbohydrate present in hemicellulose is made of xylose pentose monomers. Even though xylans are known to more susceptible to enzymatic hydrolysis when compared to cellulose, multiple xylanases with different specificities and actions are necessary to degrade the polymer (Binod et al., 2011).

The presence of arabinofuranose sidechains and methyl-glucuronic acid in the hemicellulose hinders the binding of xylanases. The arabinofuranoses are hydrolyzed by the arabinans and the methyl-glucuronic acid is hydrolyzed by α-D-Glucuronidases, which cleave the α-1,2-glycosidic bond. When the xylan is free of side chains, the endo-1,4-β-xylanase cleaves the glycosidic bond in the xylan backbone, producing xylooligomers. These oligomers then have any ester bonds, bonds crosslinking the xylan to the lignin, hydrolyzed by feruloyl esterases and any acetyl substitutions are hydrolyzed by acetyl xylan esterases. Finally, β-xylosidases hydrolyze the oligomer into xylose monomers (Shallom and Shoham, 2003).

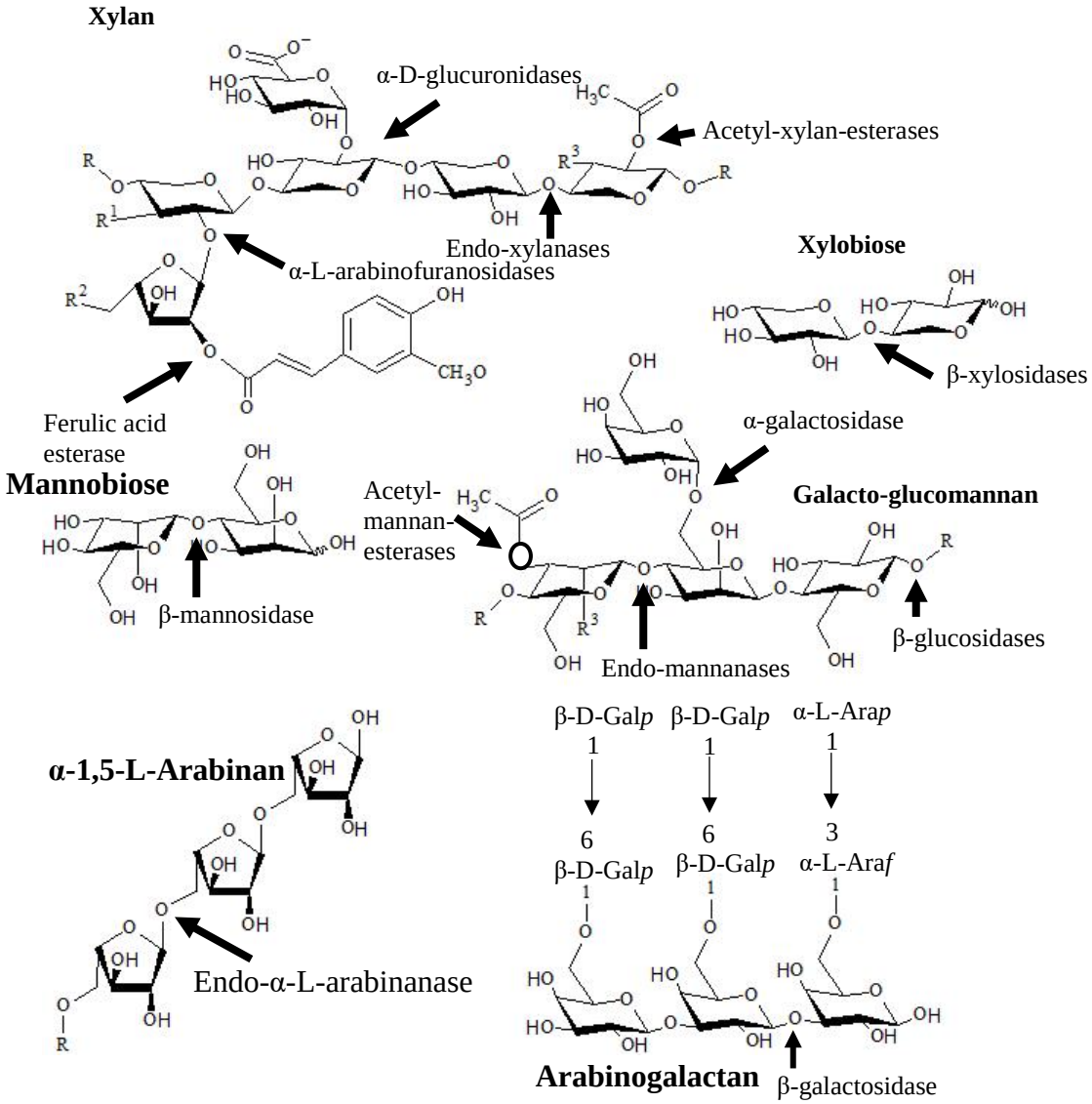

Fig. 2.6. Hemicellulases and their targeted linkages (adapted from Shallom and Shoham, 2003)

Pectins are a structural component of the plant cell wall and are common in vegetables and fruits. Pectinase is the class of enzymes that hydrolyzes pectins and is currently used in the food industry to clarify fruit juices (Gummadi and Panda, 2003). There are four types of pectinase. Pectin esterase removes methoxyl group from pectin

decreasing the gel strength. Both pectin lyase and polygalacturonase cleave the α-1,4 glycosidic bond between uronic acid monomers. The difference between the two is pectin lyase has a β-elimination mode of action, while polygalacturonases hydrolytically cleaves the bond (Bateman and Millar, 1966; Yadav et al., 2009). The final type of pectinase are polymethygalacturonases, which both remove methoxyl groups and cleave α-1,4 glycosidic bonds (Fig 2.7.) (Gummadi and Panda, 2003).

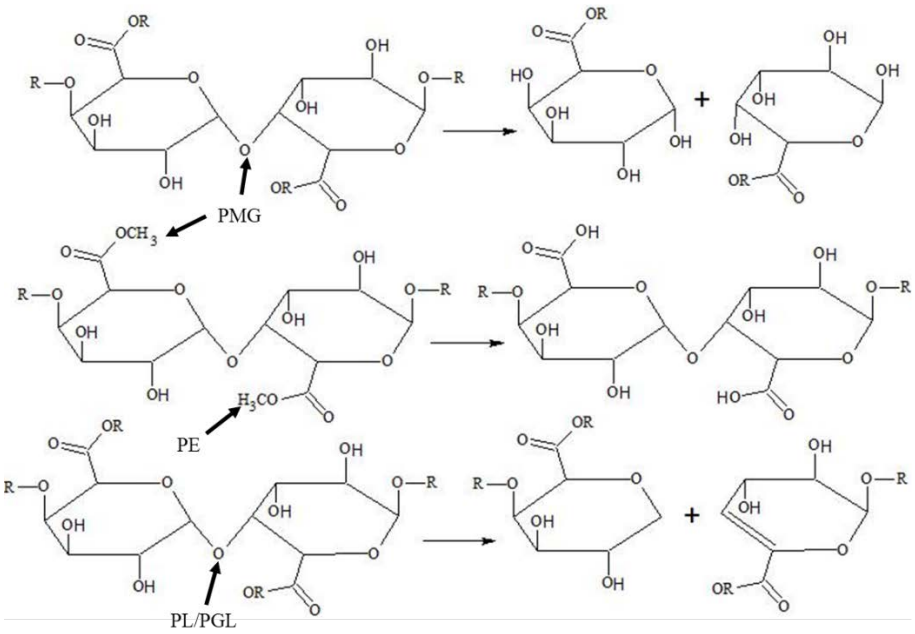

Fig. 2.7. General reactions for pectinases
PMG/PGL = polymethylgalacturonase/polygalacturonaselyase; PE = pectin esterase; PL pectin lyase (Adapted from Gummadi and Panda, 2003)

2.5.4 Effectiveness of a pretreatment

The effectiveness of a pretreatment depends the feedstock being pretreated (leaves, bark, pomaces) and the yield of the molecules of interest. Yet, a successful pretreatment is noted to have the following characteristics: avoids the need to reduce the size of biomass particles, preserves the pentose fraction, limits formation of degradation products that inhibit the growth of fermentative microorganisms, minimizes energy

demands, and limits cost (National Research Council, 2000). The value of the pretreatment product has to also be evaluated against the pretreatment's operating, capital, and biomass cost (Wyman, 1999).

2.6 Conclusion

Olive mill wastes are the residues produced from olive oil processing. The increasing worldwide demand for olive oil means an increased production of OMW, which are known for their severely deleterious environmental effects. At the same time, the compounds that cause those effects, the phenolic compounds, oleic acid, and high organic material are valuable products. Therefore, many researchers have tried to extract these compounds or convert OMW into valuable products such as animal feed or biofuels. The composition of OMW can hinder or inhibit these processes, which is why pretreating OMW is a necessary step. However, the current pretreatments used for OMW can lead to the production of even more waste or more inhibitory molecules. For that reason, novel methods and approaches are necessary to pretreat and then valorize this substrate in an environmentally friendly yet cost effective and profitable manner.

CHAPTER 3 – MATERIALS AND METHODS

3.1 Olive cake

The olive cake was obtained from La Panza Ranch, an olive oil processor located in Santa Margarita (California) and came from *Arabosana* olives in September 2016 and September 2017. The cake obtained on September 2016 was used for the biomethane and bioethanol experiments. The cake obtained on September 2017 was used for comparing the percentage of stones removed by destoning with a horizontal screw press and destoning by centrifugation. The cakes were stored in the dark at -20 °C until further processed.

3.2 Pretreatment

For all pretreatments, the olive cake was first diluted with 56 °C tap water at a 2:3 (as is) ratio. The enzymatic pretreatment involved a cocktail of four enzymes at 0.4% w/w (db, each): cellulase (powder; activity 100,000 CU/g; optimum pH 4-6.5, temperature 30-70 °C), hemicellulase (powder; activity 400,000 HCU/g; optimum pH 2-8, temperature 25-90 °C), xylanase (powder; activity 100,000 XU/g; optimum pH 3.5-6.5, temperature 40-65 °C), and pectinase (powder; activity 8,000 ENDO-PG/g; optimum pH 2-4, temperature 30-60 °C), from BIO-CAT (Troy, Virginia, USA). After enzymatic addition, diluted samples (2.5 kg) were mixed with a large spatula for 15 min and shaken in a MaxQ 5000 Floor-Model Shaker (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 24 h at 54 °C and 150 rpm. After shaking, enzymatically pretreated cakes were stored in the dark at -20 °C or immediately destoned. Fig. 3.1 summarizes the pretreatment process. Control (DOC) and destoned (DDOC) olive cakes were prepared in the same conditions without the addition of the enzyme cocktail.

For destoning, after the shaking of samples, the olive cakes were immediately processed at 500 rpm with a horizontal screw press (HSP) (Leeson, Model C145T17FB60D) equipped with a 0.71 mm mesh screen. After the first run, the liquid stream was collected and run through the HSP two more times before being stored in the dark at -20 °C until further processed. Pretreatments were done in triplicate.

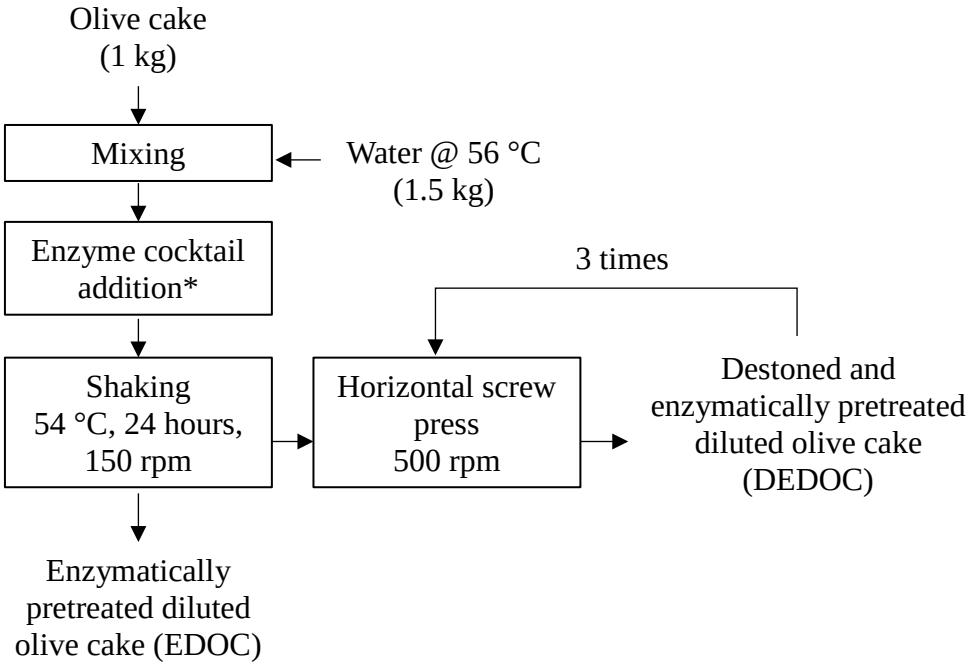

Fig. 3.1. Pretreatment flow chart
*Diluted olive cake (DOC, Control) and destoned diluted olive cake (DDOC) were obtained without enzymatic addition

3.3 Destoning by horizontal screw press (HSP) and centrifugation

An HSP was used to destone the enzymatically pretreated cake (EDOC). The same process mentioned in 3.2 was followed. The resulting olive cake was called destoned and enzymatically pretreated olive cake by horizontal screw press (DEDOC-H).

For the centrifuged samples, 950 ± 25 mL of EDOC was centrifuged in a 1 L bottle (JLA – 9.1, Beckman Coulter, Brea, California) at 100, 200, or 3,000 x g for 5 min.

The supernatant, which was decanted off, was called destoned and enzymatically pretreated diluted olive cake by centrifugation (DEDOC-C).

The percentage of stones removed was calculated by decanting a 200 g (as is) aliquot of pretreated olive cake and comparing it to the corresponding controls, which were the EDOC for the HSP and centrifugation experiments, and the sample with no pretreatment (DOC) for the comparison of destoning and enzymatic pretreatments. Stones were collected from the bottom of the container and then dried for 72 h at 100 °C before being weighed. Destoning experiments were performed in triplicate.

3.4 Analytical methods for olive cake

3.4.1 Proximate analysis

Total solids (TS) and volatile solids (VS) of the pretreated olive cakes (DOC, DDOC, EDOC, DEDOC) and inoculum were determined using standard techniques (APHA., 1985). The pH was measured with an EW-59500-81 pH electrode (Oakton, Illinois).

The olive cakes were freeze dried in a HR7000-M freeze dryer (Harvest Right, North Salt Lake, Utah) before proximate analysis. Kjeldahl nitrogen was measured with a Kjeltec 8200 (FOSS, Eden Prairie, Minnesota) and lipid content was determined with a Soxtec 2043 (FOSS, Eden Prairie, Minnesota) (AOAC., 2005). Proximate analysis was done in triplicate.

3.4.2 Reducing sugar content

The supernatant of a 1.5 mL aliquot of each sample was collected after centrifugation at 8,000 rpm for 30 min at room temperature. The reducing sugar content was measure using the dinitrosalicylic acid (DNS) method. The DNS reagent contained

1% w/v DNS, 20% 2 N sodium hydroxide, 30% w/v sodium potassium tartrate and it was adjusted to 100 mL with distilled water. DNS reagent (1 mL), water (2.5 mL), and olive cake supernatant (40 µl) were added to a test tube and boiled for 5 min. The test tubes were cooled to room temperature and the absorbance was recorded at 540 nm. The absorbance was compared to a 0-4 mg/mL glucose standard curve.

3.4.3 Total phenol extraction and analysis

The total phenols of the olive cake was determined with a modified Folin-Ciocalteau method (Obied et al., 2007). Two aliquots of the pretreated olive cakes (as is) were thawed overnight at 5 °C. The olive cake (5 g each aliquot) was extracted in a 15 mL of 80% v/v methanol:water solution for 30 min, while being continuously stirred. The resulting extract was filtered through Whatman N°1 filter paper. The solid residue was recovered from the filter paper and extracted once again with a fresh 15 mL of 80% v/v methanol:water solution for 15 min while being continuously stirred. The extract was filtered through a new Whatman N°1 filter paper before being combined with the previous extract in amber vials. The filtered extracts were defatted with 30 mL of *n*-hexane. The defatting process was repeated with 30 mL of *n*-hexane before the defatted extracts were filtered through Whatman glass fiber filter paper (Whatman GF/F ø 42.5 mm). The extract was kept in the dark at 2 °C until analysis was performed. On the day of analysis, all volumetric flasks (10 mL) were wrapped in foil to limit exposure from light. The extracts were diluted with water(1:4, extract to water) and 100 µl of the diluted extract was added to each test tube. An additional 6 mL of deionized water and 500 µl of Folin-Ciocalteu reagent were added. After 1 min, 1.5 mL of sodium carbonate solution (20% w/v) was added. Volumetric flasks were stoppered and then shaken. After 1 h at

ambient temperature, the absorbance was read at 760 nm with a Genesys 20 spectrophotometer (Thermo Fischer Scientific, Waltham, Massachusetts). The absorbance was compared to a standard curve of 0.0 mg/mL-5.0 mg/mL of gallic acid. The total phenol concentration was done in triplicate and expressed as in mg gallic acid equivalent (GAE)/g of extract.

3.4.4 Fiber analysis

Fiber analysis was done on the freeze-dried olive cake following the Van Soest method (AOAC, 1990). Amylase neutral detergent fiber (aNDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) analyses were performed sequentially. Hemicellulose was expressed as the difference of aNDF and ADF; cellulose, the difference between ADF and ADL; and lignin as the remaining material.

Berzelius beakers (600 mL) containing 0.5 g of sample and 200 mL neutral detergent solution (Ankom, Macedon, New York) were refluxed, boiled and the vapors were re-condensed into the liquor, using a Labconco crude fiber apparatus (Kansas City, Missouri) for 5 min before 2 mL of heat stable α-amylase was added. After the α-amylase addition, the contents of the beaker were refluxed for an additional 60 min. The NDF solution and residue were vacuum filtered into a fritted crucible (50 mL, coarse porosity, 40-60 μm). When only residue was left in the crucible, 50 mL of boiling deionized (DI) water and 2 mL of α-Amylase were added and allowed to set for 1 min before being filtered. The samples were then soaked in 30 mL of acetone before being filtered. After being filtered, the crucible was once again soaked in 30 mL of acetone and filtered once again. The crucibles were placed under a fume hood overnight and then placed in a 105 °C drying oven (Blue M, Blue Island, Illinois) for 24 h before ADF analysis.

Acid detergent fiber analysis followed the same procedure as aNDF. The only modifications were that α-amylase was not used and neutral detergent solution was replaced with acid detergent solution.

The final step was ADL analysis. The crucibles were filled with concentrated sulfuric acid (98%) for 3 h before being washed with boiling DI water until the pH was neutral. The samples were then dried for 24 h at 105 °C. All sequential fiber analysis was performed in triplicate.

3.5 Anaerobic digestion

3.5.1 Inoculum

The inoculum was a digested wastewater sludge from a municipal wastewater treatment facility that went through a 60 day anaerobic treatment at 35 °C (San Luis Obispo Water Resource Recovery Facility (SLO WRRF), California). It was stored in an incubator at 35 °C until used.

3.5.2 Anaerobic reactors

Biochemical methane potential (BMP) assays were conducted according to Angelidaki et al. (2009) with slight modifications. Serum bottle digesters (165 mL) were filled to a working volume of 100 mL. Triplicate control blanks containing the inoculum accounted for residual methane production due to its biodegradation. The glucose control determined the upper limit for methane yield. The substrate volatile solid (VS) loading was 7.5 g/L and the corresponding substrate:inoculum (S:I) ratio was 0.5 by VS for the glucose control and all olive cakes. No nutrients or buffers were added, and digesters were flushed with N_2 gas for a minute before being sealed with butyl septa. The digesters were incubated at 37 ± 2 °C and manually shaken for one minute every day for 30 days.

Digesters were done in duplicate but prepared in sets of three; one bottle in each set was taken apart at the mid-point, i.e., after 15 days, and their carbohydrate content was analyzed.

3.5.3 Biochemical methane potential (BMP) assay scale-up

The BMP assay scale-up was performed on destoned olive cake (DDOC) and enzymatically pretreated olive cake (EDOC), at the same S:I ratio, and under the same conditions as the initial BMP assay. Glass vessels (2.3 L) with a working volume of 1 L were used instead of the previous 100 mL working volume. Duplicate control blanks containing the inoculum accounted for residual methane production.

3.5.4 Biogas analysis

The biogas yield for each digester was volumetrically measured with a liquid displacement method using a graduated cylinder (mL biogas/day) (Walker et al., 2009). The headspace gas was collected and analyzed with a gas chromatograph (SRI 8610, Torrance, California, USA) equipped with a thermal conductivity detector (TCD) and a 1.8 m concentric packed column with a 3.18 mm inner column filled with a proprietary porous polymer mixture and a 6.35 mm diameter molecular sieve outer column (Alltech CTR1, Deerfield, Illinois). Argon was used as a carrier gas at a flow rate of 16 mL/min. The operating temperature was 55 °C, and 1 mL of sample was injected in duplicate (APHA., 1985). The percentage of the CH_4, CO_2, N_2, and O_2 in the biogas was determined by a comparison to standard gases of known purity.

Biogas of the scale-up was quantified daily for the first five days of the experiment and then taken once every two days for the remaining 25 days.

3.5.5 Calculation

The Buswell formula (Eqs. 3.1. and 3.2.) were used to calculate the theoretical methane yield (TMY, mL CH_4/g VS) for the glucose control. The letters n, a, and b equal to 6, 12, and 6, respectively (Li et al., 2013).

$$C_nH_aO_b + (n - \frac{a}{4} - \frac{b}{2})\ H_2O \rightarrow (\frac{n}{2} - \frac{a}{8} + \frac{b}{4})\ CO_2 + (\frac{n}{2} + \frac{a}{8} - \frac{b}{4})\ CH_4 \quad (3.1.)$$

$$TMY = [22.4 \times 1000 \times (\frac{n}{2} + \frac{a}{8} - \frac{b}{4})]/(12n + a + 16b) \quad (3.2.)$$

Using Eq. 3.2. the TMY of glucose was 373.5 mL CH_4/g VS at standard temperature and pressure.

Specific methane yield (mL CH_4/g VS) is the normalized amount of methane produced by the substrate. Specific methane yield was calculated by subtracting the methane yield of the inoculum blank (mL CH_4 inoculum) from the methane yield of each substrate (mL CH_4 substrate). The resulting value was divided by the mass of the substrate VS added to the digester (Eq 2.3).

$$SMY = (mL\ CH_4\ substrate - mL\ CH_4\ inoculum)/(g\ VS\ substrate\ added) \quad (3.3.)$$

A modified Gompertz equation (Eq. 3.4.) (Serrano et al., 2017) was used to fit the data through non-linear regression using Microsoft Excel's Solver tool. Fit was evaluated by the R – squared (R^2) values.

$$Y(t) = Y_m \times e^{\{-e^1 \times \left[\frac{R_m \times e^1}{Y_m}(\lambda - t) + 1\right]\}} \quad (3.4.)$$

Y(t) describes the cumulative SMY at time *t* (mL CH_4/g VS), Y_m is the ultimate cumulative SMY (mL CH_4/g VS), R_m refers to the maximum methane yield (mL CH_4/(g VS d)), λ refers to the lag phase duration (d), and *t* refers to time in days (d). The time when R_m is reached is known as t_{max} (d) and was calculated with the following equation (Eq. 3.5.):

$$t_{max} = \lambda + \frac{Y_m}{R_m \times e^1} \quad (3.5.)$$

3.5.6 Carbohydrate profile

Characterization and quantification of the carbohydrate monomers were performed before digestion and on the digestate on days 0, 15, and 30 with an Aminex HPX-87H column (Bio-Rad, Hercules, California) in a Prominence Ultra Fast Liquid Chromatograph (UFLC) (Shimadzu, Kyoto, Japan) equipped with an Agilent 1200 series Refractive Index (RI) detector (Santa Clara, California). The mobile phase was 0.005 M sulfuric acid running at an isocratic flow rate of 0.6 mL/min. Glucose, xylose, galactose and arabinose at concentrations of 0.125, 0.25, 0.5, and 1 mg/mL were used as standards. In these conditions, galactose and xylose had the same retention time and were not differentiated. Supernatant was collected from the pretreated olive cakes and was diluted ten times with nanopure water (Thermo Fischer Scientific, Waltham, Massachusetts). Supernatants collected after the olive cake had been combined with the inoculum on days 0, 15, and 30 were not diluted. All samples were filtered through a 25 mm syringe with a 0.45 µm polyvinylidene fluoride membrane (Thermo Fischer Scientific, Waltham, Massachusetts) before 10 µL was injected into the UFLC in duplicate. Peaks were integrated with the LabSolutions Analysis Data System (Shimadzu, Kyoto, Japan).

3.5.7 Analytical methods for digestate of the scale-up BMP

At the end of the 30-day digestion for the scale-up experiment, the digestates from the destoned olive cake (DDOC) and enzymatically pretreated olive cake (EDOC) were dried in a convection oven at 50 ± 5 °C for 72 h. Kjeldahl nitrogen, lipid content, sequential fiber analysis, and total phenols followed the same methods mentioned above and were performed in duplicate.

3.5.8 Gross heat of combustion

Gross heat of combustion was measured in duplicate using a Parr 1241 Oxygen Bomb Calorimeter and 1180 Oxygen Combustion Bomb (Moline, Illinois, United States). Methods N°203M were applied (Parr Instrument Company, 2013). The procedure followed the operating instruction manual for both the calorimeter and the bomb. A Parr pellet press was used to compress 0.5 g of residue before the analysis. Gross heat of combustion was performed in duplicate and expressed as kJ/g TS.

3.6 Ethanol fermentation

Fermentations were done in 250 mL round bottom flasks in a Tornado System (Radleys, Essex, UK). Five grams (db) of olive cake (DOC, DDOC, EDOC, or DEDOC) was added to a flask which was docked in a Carousel 6 Plus Reaction Station™. Lactrol (2 mg) was dissolved in 1 L of 0.05 M citrate buffer at pH 5. The citrate buffer was added to the round bottom flasks so there would be a working mass of 117 g. Two grams of dry yeast (Ethanol Red, Lesaffre Advanced Fermentation, Durham, North Carolina), 1 g of yeast extract, and 2 g of peptone were mixed in 100 g of deionized water at 35 °C for 30 min. Thirteen grams of this mixture was added to each flask. The pH of the contents of the round bottom flask was measured using an Orion 3-star Benchtop pH meter (Thermo Fisher Scientific, Waltham, Massachusetts) and adjusted using 0.1N NaOH or 0.1N HCL until it was 5.0 ± 0.1.

The temperature of the Tornado system was set to 35 °C using a heating plate, MR Hei-Tec (Heidolph, Schwabach, Germany). The RZR 2052 Control (Heidolph, Schwabach, Germany) ensured stirring at 200 rpm. The shafts used for stirring were Polytetrafluoroethylene (PTFE) anchors purchased from Radley.

Samples for HPLC-RI analysis were taken at 0 and 72 h. At either time, a sample of 10 mL was collected in a 15 mL Falcon tube and stored at 2 °C until analysis. At the end of the experiment, the samples were centrifuged (mySpin 12 Microcentrifuge, Thermo Fischer Scientific, Waltham, Massachusetts) at 8,000 rpm for 30 min and the supernatant was collected in an Eppendorf tube. The supernatant was then filtered through a 0.2 µl syringe filter. The HPLC procedure followed Section 3.3.3 with the following modifications: the supernatant (1 mL) was diluted with 9 mL of nanopure water. A standard curve ranging from 0.125 mg/mL-1 mg/mL was created with glucose, xylose, arabinose, lactic acid, acetic acid, and ethanol (Fig. 3.2). The analysis was done in triplicate.

The theoretical ethanol yield was calculated as the percentage of the ethanol produced (mg/mL) in 72 h and the concentration of glucose consumed (mg/mL) in the same amount of time multiplied by the stoichiometric yield of ethanol from glucose (0.511g ethanol/g glucose) (Eq. 3.6.).

$$\text{Theoretical ethanol yield} = \frac{(\text{Ethanol}_{t72} - \text{Ethanol}_{t0})}{(\text{Glucose}_{t0} - \text{Glucose}_{t72}) \times 0.511} \quad (3.6.)$$

3.7 Statistical analysis

Average values were reported. JMP software was used for analysis of variance (ANOVA) and to perform Tukey's Honest Significant Different (HSD) test with a critical significance level of 0.05.

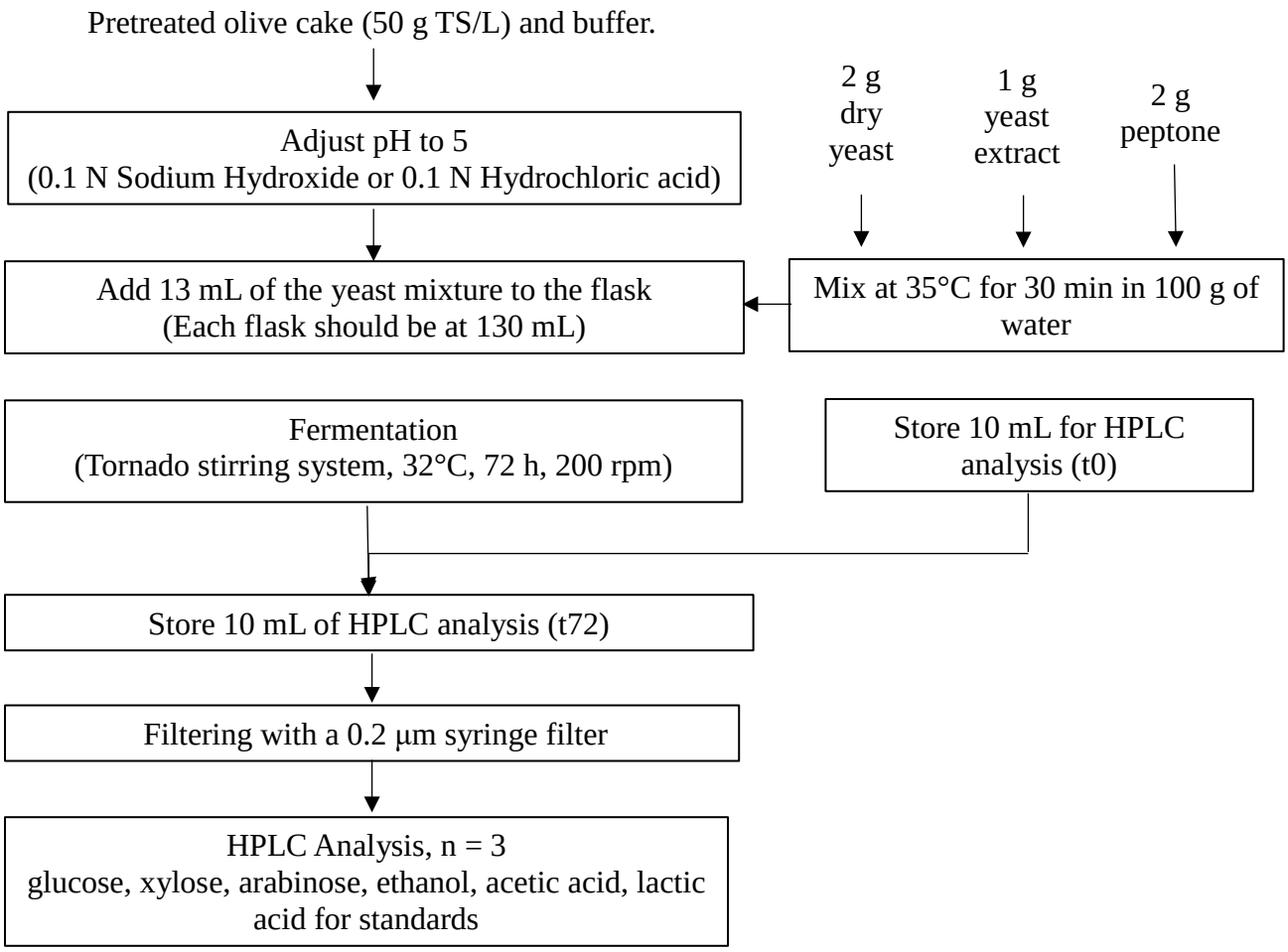

Fig. 3.2. Ethanol process flow chart

CHAPTER 4 – RESULTS AND DISCUSSION

4.1 Stone removal efficiency with horizontal screw press (HSP) and centrifuge

The comparison of destoning by HSP and centrifuge was performed on the enzymatically pretreated diluted olive cake (EDOC). These mechanical pretreatments were done to remove as many of the stone fragments as possible, while retaining the maximum amount of the digestible total solids in the liquid fraction for subsequent anaerobic digestion.

Olives stones represent 10-30% of the mass of the olive cake (Hernández et al., 2014). Data on the percentage of stones removed after industrial destoning by cyclone separation is scarce. Through the use of cyclone separation, the quantity of olive stone fragments obtained range from 25-40% of the weight of the olive cake (Barreca and Fichera, 2013). The quantity of stones obtained from the horizontal screw press was 46% (db) of the weight of the olive cake. Though, the quantity of olive stones obtained varies due to extraction method and olive cultivar. Thus, the percentage of stones removed, the amount of stones removed by destoning divided by the amount of stones in the control (EDOC), was used to compare the stone removal efficacy of the HSP and centrifuge.

Both the HSP and centrifuge, regardless of the centrifugal speed, removed over 95% of the stones (Fig. 4.1). A lower stone removal percentage of 84% was reported after steam explosion treatment at 135 s at 234 °C or 135 s at 193 °C (Felizón et al., 2000). Unlike destoning by HSP or centrifuge, steam explosions require considerable amounts of energy, limiting its use as an environmentally friendly pretreatment.

The distribution of total solids (TS) between the solid and liquid fractions during separation showed that at 3,000 x g, less than a quarter (21%) of the total solids of the

olive cake was recovered in the liquid fraction (Fig. 4.1). At a centrifugal speed of 200 x g, 27% TS remained in the liquid fraction. Lowering the speed to 100 x g significantly increased the TS in the liquid fraction to 43%. Serrano et al. (2017) observed a similar trend using two phase olive mill waste (2POMW) pretreated at 170 °C for 60 min. When centrifuged at 4,700 x g, 18% of the total solids was retained in the liquid phase. Alternatively, the use of a horizontal screw press resulted in the highest quantity of the total solids of the olive cake (66%) in the liquid fraction. Therefore, all subsequent destoning in this study was done by horizontal screw press.

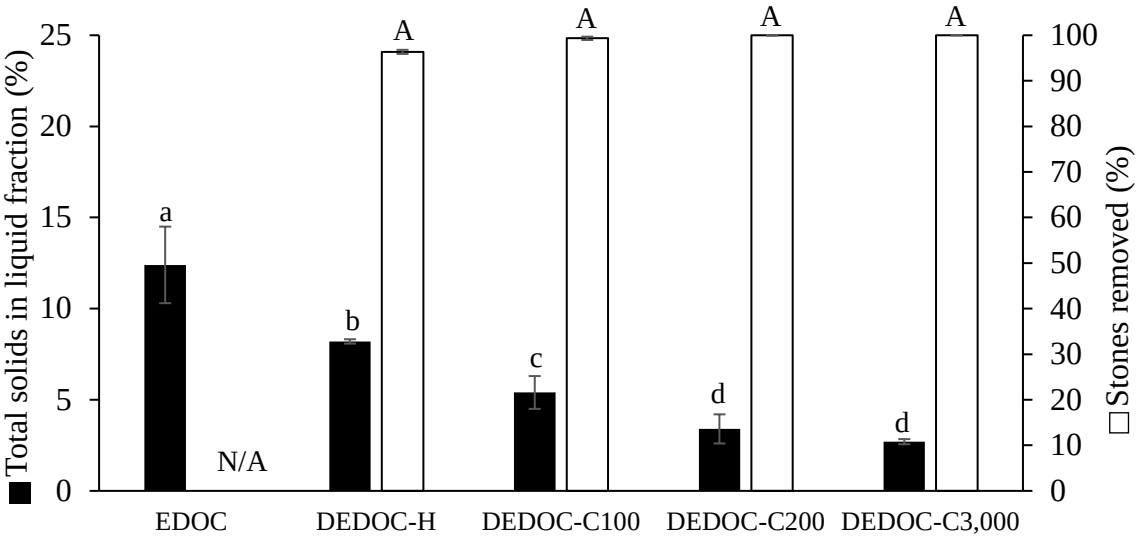

Fig. 4.1. Comparison of the percentage of stones removed with a centrifuge and a horizontal screw press on enzymatically pretreated diluted olive cake
Different letters within a group indicates significant differences. N/A = not applicable; Control for stones removed (%) was EDOC. Enzymatically pretreated diluted olive cake (EDOC); destoned enzymatically pretreated diluted olive cake by horizontal screw press (DEDOC-H); destoned enzymatically pretreated diluted olive cake by centrifugation at 100 x g (DEDOC-C100); destoned enzymatically pretreated diluted olive cake by centrifugation at 200 x g (DEDOC-C200); destoned enzymatically pretreated diluted olive cake by centrifugation at 3,000 x g (DEDOC-C3,000)

The difficulty in using a horizontal screw press for destoning olive cake is that olive cake must be diluted with water before it can flow through the machine. The HSP required 150 g tap water/100 g of olive cake. One alternative to the use of tap water to

diluted olive cake is using olive mill wastewater that has been stripped of its phenolic compounds. Not only is OMWW capable of diluting olive cake so it can go through the HSP, the OMWW also has a large amount of digestible organic matter (40-220 g/L) (Niaounakis and Halvadakis, 2006a). Further research and an economic analysis of this process is recommended as using OMWW in this manner simultaneously valorizes both olive waste products.

4.2 Comparison of stone removal by horizontal screw press (HSP) vs. enzymatic pretreatment

When the HSP was applied to the diluted control (DOC), a 94% reduction in stone content was obtained in the DDOC (Fig. 4.2). Interestingly, it was observed that even though the enzymatically pretreated olive cake (EDOC) was not submitted to the destoning process, its stone content was 31% lower than the control (DOC). This observed mass reduction was attributed to the loss of attached hemicellulose and cellulose to the stones due to the enzymatic hydrolysis. This claim was supported by comparing the TS contents of the DOC (19.2%) and EDOC (20.1%) ($p > 0.05$), demonstrating the compounds formed during enzymatic hydrolysis stayed in the liquid fraction.

The effect of soaking time (1 h vs 24 h) before the control olive cake was destoned (DDOC) was not significant, indicating that an additional 23 h soak at 54 °C did not further solubilize hemicellulose and cellulose around the stones (Fig. 4.2). This result is of significance as it shows that 1 h of soaking at 54 °C is enough for optimal destoning. The effect of no soaking (0 h) was not investigated. After the addition of tap water to the

olive cake, the sample was mixed for 15 minutes. Without this mixing, the olive cake would not run through the HSP.

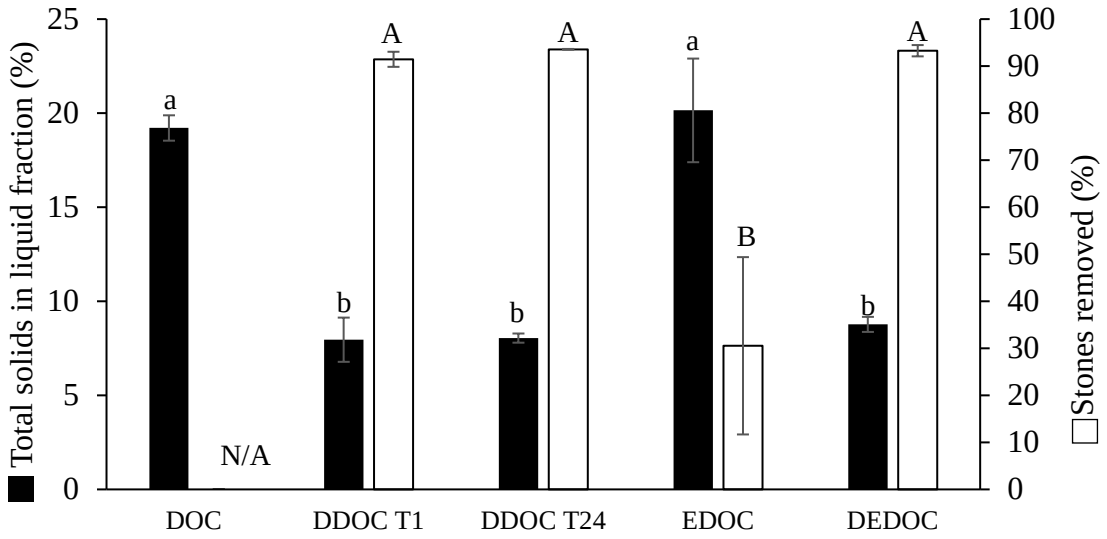

Fig. 4.2. Percentage of stones removed with a horizontal screw press on diluted olive cake and enzymatically pretreated diluted olive cake
Different letters within a group indicates significant differences. N/A = not applicable; Control for stones removed (%) was DOC. Diluted olive cake (DOC, control); destoned diluted olive cake soaked for 1 h and 24 h (DDOC T1 and DDOC T24, respectively); enzymatically pretreated diluted olive cake (EDOC); destoned and enzymatically pretreated diluted olive cake (DEDOC)

4.3 Impact of pretreatment on olive cake characteristics

Table 4.1 summarizes the physical and chemical properties of the olive cakes used in this study. Olive cake is known to have low amounts of protein (3.3-9.6%, db) (Chiofalo et al., 2004; Ferrer et al., 2018). After applying a Kjeldahl-nitrogen-to-protein-conversion-factor of 6.25 (Kailis and Harris., 2007), the olive cake in our study had a protein content of 4.6% (db). The removal of stones concentrated the pulp, increasing the amount of protein to 8.9%, which confirmed results reported in previous studies when commercial destoned cake was compared to crude ones (Chiofalo et al., 2004; Sadeghi et al., 2009).

Table 4.1. Characteristics of control and pretreated olive cakes and inoculum

Characteristics	Inoculum	DOC[1]	DDOC[2]	EDOC[3]	DEDOC[4]
Total Solids (g/kg)	26.1 ± 0.1[a]	192.0 ± 6.7[c]	80.0 ± 2.4[b]	201.0 ± 27.5[c]	87.0 ± 4.0[b]
Kjeldahl Nitrogen (%TS)	N.D.	0.8 ± 0.1[a]	1.4 ± 0.1[b]	0.9 ± 0.2[a]	1.6 ± 0.1[b]
Crude Fat (%TS)	N.D.	8.3 ± 0.3[a]	13.9 ± 0.5[c]	10.6 ± 1.0[b]	10.4 ± 0.8[b]
Volatile Solids (%TS)	62.4 ± 0.0[a]	97.0 ± 0.3[c]	92.0 ± 0.3[b]	97.0 ± 0.4[c]	92.0 ± 0.4[b]
Ash (%TS)	37.6 ± 0.2[c]	3.0 ± 0.4[a]	8.2 ± 0.3[b]	3.2 ± 0.5[a]	7.8 ± 0.4[b]
pH	7.9 ± 0.0[b]	4.3 ± 0.2[a]	4.3 ± 0.1[a]	4.2 ± 0.2[a]	4.1 ± 0.1[a]
Hemicellulose (%TS)	N.D.	22.9 ± 3.7[b]	16.3 ± 1.1[a]	22.0 ± 3.3[b]	15.1 ± 2.0[a]
Cellulose (%TS)	N.D.	15.0 ± 3.5[b]	12.0 ± 0.4[ab]	14.5 ± 2.5[b]	10.0 ± 1.5[a]
Lignin (%TS)[5]	N.D.	29.0 ± 2.9[b]	12.1 ± 1.0[a]	24.4 ± 5.2[b]	14.0 ± 1.6[a]
Total Phenolics (mg GAE/g)[6]	N.D.	0.7 ± 0.1[a]	0.8 ± 0.2[a]	1.0 ± 0.1[a]	0.9 ± 0.2[a]
Reducing Sugar (mg/mL)	N.D.	10.0 ± 2.0[a]	11.8 ± 3.8[a]	27.9 ± 3.9[b]	27.3 ± 1.3[b]

Different letters for each characteristic indicates a significant difference. 1. Diluted olive cake, control; 2. Destoned diluted olive cake; 3. Enzymatically pretreated diluted olive cake; 4. Destoned and enzymatically pretreated diluted olive cake; 5. Acid detergent lignin; 6. mg GAE/g = mg gallic acid equivalent/g of extract; TS = Total solids (dry matter); N.D = not determined

Residual oil content in olive cakes depends on the efficiency of olive oil extraction. Fat levels can be as low as 7.3% (db) or as high as 22.1% (db) (Vlyssides et al., 1998; Chiofalo et al., 2004). In our study, the olive cake contained 8.3% fats (db). Both destoning and enzymatic pretreatment increased fat content. Fat content in olive cake may be underestimated due to the low percolation, the rate at which the solvent passes through a permeable substance, of hexane through the olive cake matrix (Moral and Méndez, 2006). Enzymatic pretreatment is capable of decreasing the particle size of the olive cake, increasing the solvent penetration, which thereby facilitates better oil extraction.

As expected, the reducing sugar content was increased after enzymatic pretreatment. The amount of digestible carbohydrates immediately available for

biomethane or bioethanol production increased by almost a factor of 3 (DOC, 10.0 mg/mL vs EDOC, 27.9 mg/mL). Total phenol concentrations (0.7-1.0 mg GAE/g) of all four olive cakes were similar to the ones reported by Leouifoudi et al. (2015) for various olive varieties. This concentration was well below the demonstrated inhibitory level of 1.7 mg GAE/g for unadapted methanogenic microorganisms (Akassou et al., 2010).

Acid detergent lignin in olive cake ranges from 12-30% (db) depending on the variety of the olives (Chiofalo et al., 2004; Ferrer et al., 2018). After destoning with the HSP, lignin content decreased more than 50% (DOC, 29.0% vs DDOC, 12.1%), which was higher than the 25% decrease in lignin when destoning by a densimetric method (Hachicha et al., 2003). A large reduction in lignin after destoning can be explained by the phytotomy of olives. Classical lignocellulosic biomasses such as corn stover have cellulose in a crystalline form that is cross-linked with hemicellulose and lignin polymers. This lignin acts as a cement, restricting enzyme access to the cellulose. Such a decrease in the enzymatic accessibility of polysaccharides is less of a problem in olive cake as a large portion of the lignin is localized in the kernel and stone, while the flesh is composed of a cellulose matrix. The closer the pulp is to the stone, the higher its lignin content (Coimbra, 1994). Thus, the removal of olive stones is an efficient way to remove lignin. Lignin can be further converted into phenolic compounds using laccase and lignin peroxidases, lignin degrading enzymes. The antioxidant potential of lignin degradation products from enzymatic conversion has yet to be investigated (Davis et al., 2016).

4.4 Methane yields

The efficiencies of the pretreatments on the specific methane yield were determined with the biochemical methane potential (BMP) assay, which has been used as standard protocol to estimate the specific methane yield (SMY) of solid agricultural residues (Angelidaki et al., 2009). Olive cake's conversion to biomethane has been scarcely investigated, mostly because it is a good substrate for heat recovery, when GHG emissions are not considered (Masghouni and Hassairi, 2000).

The methane production from the olive cakes generally followed the same trend regardless of pretreatment: a short lag phase, followed by a steady production of methane, before plateauing on day 23 (< 5% increase for 7 days) (Fig. 4.3).

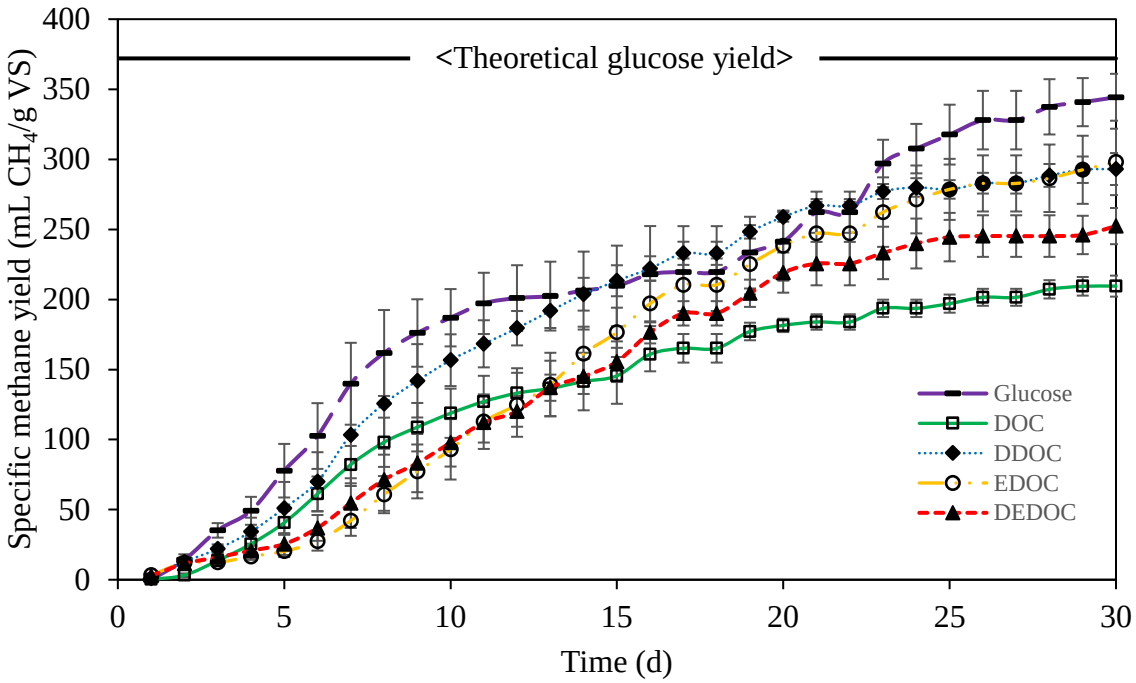

Fig. 4.3. Effect of enzymatic pretreatment and destoning on specific methane yield DDOC and EDOC are not statistically different at day 30. A plateau started at day 23 (< 5% increase for 7 days). Diluted olive cake (DOC, control); destoned diluted olive cake (DDOC); enzymatically pretreated diluted olive cake (EDOC); destoned and enzymatically pretreated diluted olive cake (DEDOC)

On day 30, the glucose control produced 92% of the TMY of glucose (373.5 mL CH_4/g VS), setting up an upper limit for the four olive cakes. As expected, the control (DOC) displayed the lowest SMY value (209.5 mL CH_4/g VS). The enzymatically pretreated olive cake (EDOC, 298.1 mL CH_4/g VS) and the destoned olive cake (DDOC, 293.1 mL CH_4/g VS) produced the highest amount of methane, 42% higher than that of the control (DOC) and just 14% lower than the glucose control. They were not found to be statistically different, suggesting destoning was just as effective as the enzymatic pretreatment in increasing SMY. Surprisingly, the combination of destoning and enzymatic pretreatments, i.e. DEDOC (252.3 mL CH_4/g VS) led to a 20% higher SMY than the control, but this yield was lower than the olive cakes that underwent a single pretreatment. Oleuropein and its degradation products could be the reasons for such result. Polysaccharides form complexes with polyphenols in olives, and when enzymes degrade the polysaccharides, the polyphenols are then released and degraded (Vierhuis et al., 2001). Oleuropein, known as the main phenolic compound in olive, has shown antimicrobial properties, but its degradation products such as hydroxytyrosol and elenolic acid are stronger, inhibiting microbial growth at lower concentrations (Fleming et al., 1973). Combined destoning and enzymatic treatment might have led to conditions promoting the presence of a higher amount of degradation products, while leaving the total polyphenol content of the DEDOC similar to the one of the control, resulting in a lower SMY.

To evaluate the stability of the anaerobic digestion process, both pH and alkalinity were measured after 30 days (Fig. 4.4). With our S:I ratio of 0.5, pH values were within the recommended bounds for methanogenic activity (pH 6.8 – 7.8) for all digesters (Chen

et al., 2015). At this low S:I ratio the inoculum not only diluted the olive cake but also acted as a buffer (Alzate et al., 2012). An alkalinity ranging from 8,800-9,333 mg $CaCO_3$/L was measured in all digesters. This alkalinity reflected a high concentration of calcium carbonate, which plays the role of buffer in anaerobic digester (Ruggeri et al. 2015). With a high alkalinity and pH within the recommended limits of anaerobic digestion, there was no evidence of inhibition due to acidification.

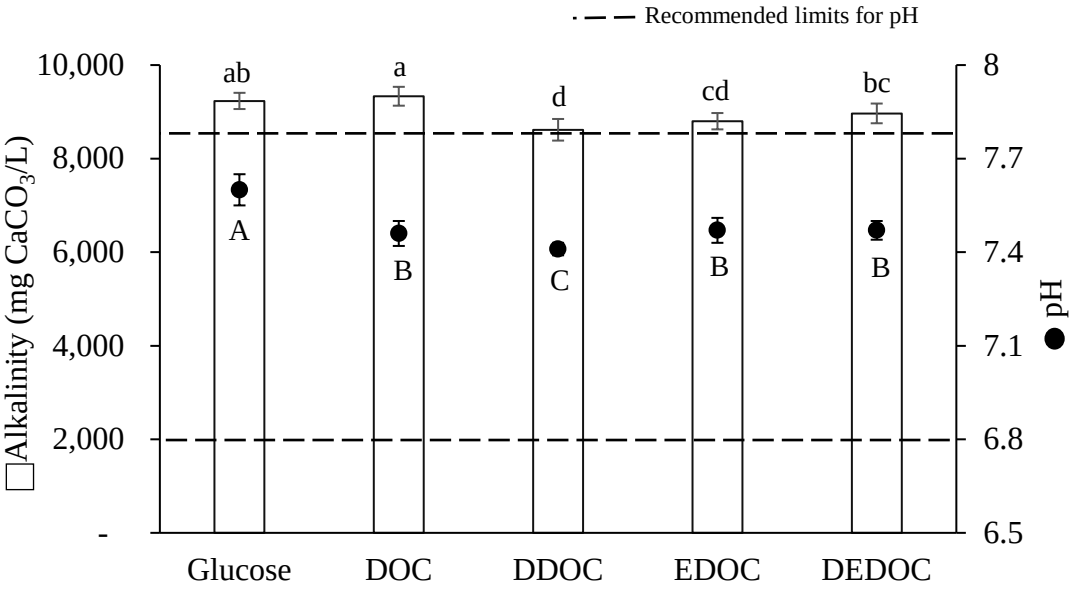

Fig. 4.4. Anaerobic digester conditions at the end of the biochemical methane potential (BMP) assay
Different lower-case letters represent significant statistical differences in alkalinity. Different upper-case letters represent significant differences in pH. Diluted olive cake (DOC); destoned diluted olive cake (DDOC); enzymatically pretreated diluted olive cake (EDOC); destoned and enzymatically pretreated olive cake (DEDOC)

Tekin and Dalgıç (2000) obtained approximately 21 mL CH_4/g VS from olive cake after a 14 day digestion period using a S:I ratio of 2 by VS with a working volume of 1 L. As aforementioned, our control (DOC) produced 209 mL CH_4/g VS after the same digestion period with a S:I ratio of 0.5 by VS. The low specific methane yield (SMY) obtained with a S:I ratio of 2 by VS used in Tekin and Dalgıç's study can be

explained by excess substrate inhibiting the induction of microbial enzymes that aid during the digestion, leading to the build-up of toxic metabolites (Grady, 1985). Another study reported a methane production of 69.8 mL CH_4/g with olive cake, and 135.4 mL CH_4/g when olive cake was co-digested with olive mill waste water (OMWW) (Ergüder et al., 2000). The former results are difficult to compare with other studies, as the S:I ratio was not reported. Similarly, in the study of Ruggeri et al. (2015) olive cake was co-digested with OMWW. Using a working volume of 100 mL at a S:I ratio of 8.7 by VS for 60 days, 1.8 mL CH_4/g VS was reported for the control, which was ~100 times less of what we obtained with DOC. After diluting the olive cake mixture with an unknown amount of tap water, the SMY increased to 103.6 mL CH_4/g VS, and adding calcium carbonate (buffering agent) further increased this value to 193.4 mL CH_4/g VS.

The S:I ratio chosen for our study (0.5 by VS) has become standard in BMP assays dealing with lignocellulosic substrates due to its suitability for producing methane (Chynoweth et al., 1993; Alzate et al., 2012). Table 4.2 compares multiple BMP assays performed in mesophilic conditions with a S:I ratio of 0.5. The theoretical methane yields (TMY) for carbohydrates, proteins, and lipids have also been included. Without any pretreatment, olive cake was found to be a reasonable candidate for anaerobic digestion, producing similar amounts of methane to two phase olive mill waste (213.1 mL CH_4/g VS) and cotton gin waste (235.7 mL CH_4/g VS). Substrates with traditionally high amounts of lignocellulosic material are generally inefficient at producing methane (pine, 59 mL CH_4/g VS). After enzymatic pretreatment or destoning, olive cake reached similar yields to nitrogen-rich substrates like chicken manure (295 mL CH_4/g VS) and higher yields than that of corn stover (241 mL CH_4/g VS).

Table 4.2. Specific methane yields for different substrates

Substrate	Specific methane yield (mL CH4/g VS)	References
DOC[1]	209.5 ± 7.5	N/A
EDOC[2]	298.1 ± 23.7	N/A
Carbohydrate (Theoretical)	350	Wilke, 2008
Proteins (Leucine, Theoretical)	570	Wilke, 2008
Fats (Lauric Acid, Theoretical)	950	Wilke, 2008
Bamboo	16	Chynoweth et al., 1993
Pine	59	Chynoweth et al., 1993
Sorghum	260 - 380	Chynoweth et al., 1993
Cellulose	370	Chynoweth et al., 1993
Food Waste	540	Chynoweth et al., 1993
2POMW[3]	213.1	Pellera and Gidarakos, 2016
Cotton Gin Waste	235.7	Pellera and Gidarakos, 2016
Juice Waste	446.0	Pellera and Gidarakos, 2016
Winery Waste	446.2	Pellera and Gidarakos, 2016
Corn Stover	241	Li et al., 2013
Vinegar Residue	253	Li et al., 2013
Rice Straw	281	Li et al., 2013
Chicken Manure	295	Li et al., 2013

1. Diluted olive cake, control; 2. Enzymatically pretreated diluted olive cake; 3. Two-phase olive mill waste

4.5 Kinetic modelling

The modified Gompertz equation models microbial activity to estimate methane production. This equation was an accurate fit for all olive cakes (all $R^2 \geq 0.99$) (Fig 4.5 (a) and (b)). The values of the parameters, Y_m, R_m, λ, and T_{max} were shown in Table 4.3. The difference between the experimental methane yields and the yields predicted by the model (Y_m) were between 0.2-2.1%.

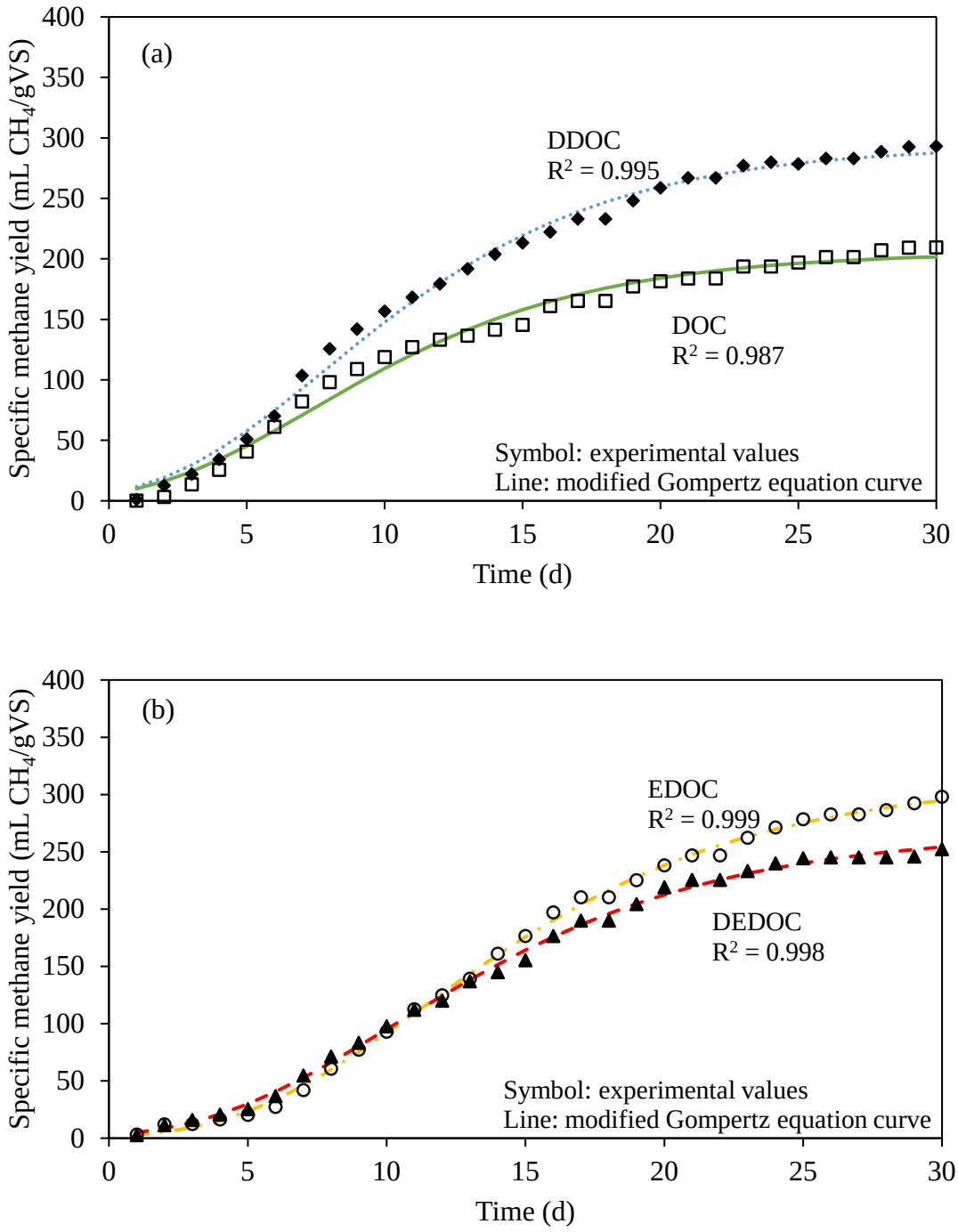

Fig. 4.5. Estimation of specific methane yield by modified Gompertz equation
(a) Diluted olive cake (DOC, control); destoned diluted olive cake (DDOC); (**b)** enzymatically pretreated diluted olive cake (EDOC); destoned and enzymatically pretreated olive cake (DEDOC)

Table 4.3.
Kinetic parameters of olive cakes estimated by modified Gompertz equation

Parameter	DOC[1]	DDOC[2]	EDOC[3]	DEDOC[4]
Y_m (mL CH_4/g VS)	205.6	294.0	313.2	267.6
R_m (mL CH_4/(g VS d)	13.2	18.6	17.3	14.8
λ (d)	1.6	2.0	4.7	3.6
t_{max} (d)	7.3	7.8	11.4	10.2
R^2	0.987	0.995	0.999	0.998

1. Diluted olive cake, control; 2. Destoned diluted olive cake; 3. Enzymatically pretreated diluted olive cake; 4. Destoned and enzymatically pretreated diluted olive cake; Y_m = expected ultimate cumulative specific methane yield; R_m = maximum methane yield; λ = lag phase duration; t_{max} = time until R_m is reached

The higher lag phase (λ) for the enzymatically pretreated olive cakes (EDOC and DEDOC) indicated that the inoculum needed a longer period of time to initiate substrate consumption. Lag phase lengths are known to be dependent on the microbial composition of the inoculum, the physio-chemical properties of the substrate, as well as how well the microbes acclimate to a new environment (Gonzalez-Estrella et al., 2017). Typically, an increased lag phase is due to the accumulation of volatile fatty acids (VFA) and a pH drop in the digester, which lowers methanogenic activity (Wilke, 2008). The pH values of our digesters were well within the recommended bounds for anaerobic digestion. There was also a sufficient amount of alkalinity to offset an accumulation of VFA (Fig. 4.4).

Commercial carbohydrases are commonly used in industrial olive oil production to hydrolyze polysaccharides during the malaxation step to increase olive oil yield (Ranalli and Serraiocco, 1996). The second possible explanation is that when the carbohydrase cocktail hydrolyzed the polysaccharides that make up the olive cake, the residual oil became more available to the anaerobic microorganisms. This residual oil is known to be high in oleic acid. While its content in olive cake has not been reported, oleic acid makes up about 75% of the fat in 2POMW (Dal Bosco et al., 2010). Low

concentrations of oleic acid (0.2 mg/mL) can increase the lag phase of anaerobic microorganisms by 10 days under thermophilic conditions and even lower concentrations have been reported to show inhibition under mesophilic conditions (Angelidaki and Ahring, 1992). It is hypothesized that a higher availability of oleic acid in the EDOC and DEDOC contributed to the additional lag. To validate this hypothesis, the fatty acid composition of the olive cakes should be characterized and quantified by GC-FAME (Chiofalo et al., 2004).

4.6 Methane composition

The composition of the biogas was 60-70% methane from day 13 onwards, and carbon dioxide was the only other component detected in the biogas (Fig. 4.6). The methane content obtained in this study was higher than the average methane composition of biogas (50-65% methane) (Kavuma, 2013).

Biogas composition can be estimated by the Buswell formula and is dependent on the chemical composition of the substrate. Pure carbohydrates produce biogas that is 50% methane while the anaerobic digestion of proteins leads to biogas composed of 55% methane. The anaerobic digestion of triglycerides lead to biogas composed of 70% methane, which is the theoretical maximum amount of methane in biogas. In spite of this theoretical limit, carbon dioxide is more than twenty times more soluble than methane in water at 30 °C (Geventman, 1999). Therefore, as more CO_2 is dissolved into the liquid phase of the digester, a larger percentage of the headspace gas of the digester is composed of methane. Due to this phenomenon, it is possible to reach methane concentrations in the headspace that are higher than 70%. In fact, biogas captured from dairy lagoons can be composed of 90% methane due to the vast amount of water (Krich

et al., 2005). The absence of a significant difference in methane composition between the olive cakes illustrated that mechanical and enzymatic pretreatments did not impact the composition of biogas produced.

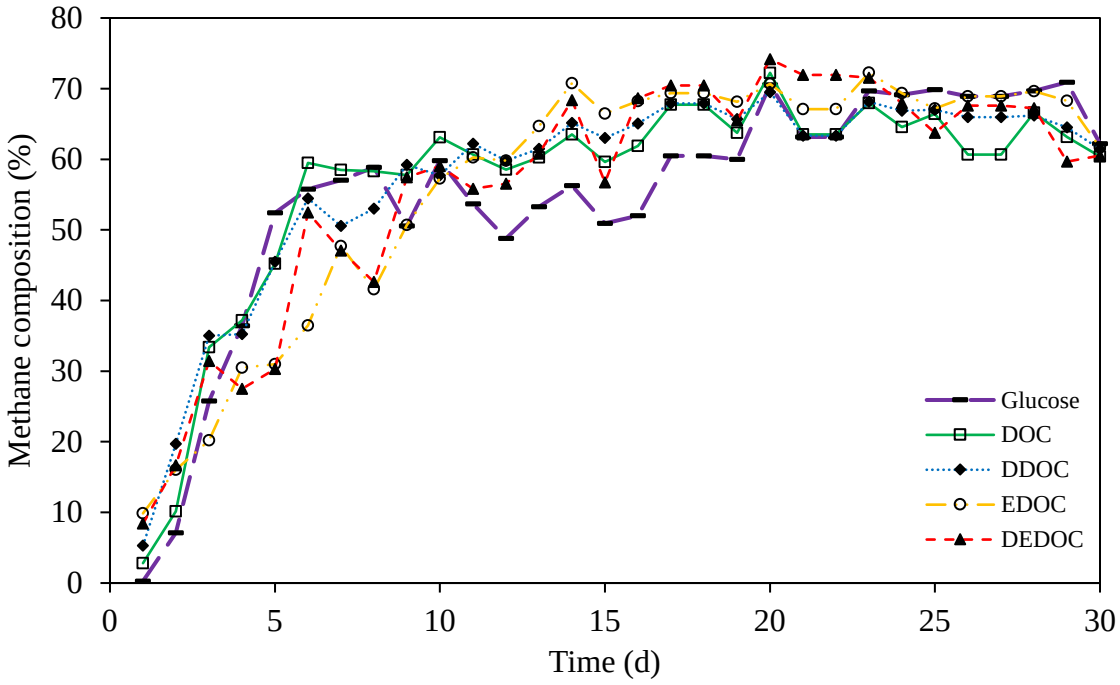

Fig. 4.6. Methane composition of pretreated olive cakes and glucose
Methane composition of all olive cakes after day 13 are not statistically different. Diluted olive cake (DOC, control); destoned diluted olive cake (DDOC); enzymatically pretreated diluted olive cake (EDOC); destoned and enzymatically pretreated diluted olive cake (DEDOC)

Another factor that influences the methane composition of the biogas is the S:I ratio. Tekin and Dalgıç, (2000) were able to produce biogas that consisted of 10% more methane than our experiment after 13 days of digestion with a S:I ratio of 2 by VS (80% methane). Our lower methane concentration was expected as digestion with a lower S:I ratio typically results in a lower percentage of methane in the biogas, even if a higher SMY is obtained. Similarly, when corn stillage was digested at a S:I ratio of 2.17 by VS, the ultimate percentage of methane in the biogas was 65% and the SMY was 691 mL

CH_4/g VS. When it was digested at a S:I ratio of 0.21 by VS the percentage of methane in the biogas had decreased by 19% to 45%, even if the SMY was 788 mL CH_4/g VS (Eskicioglu and Ghorbani, 2011). This trend is consistent up to higher S:I ratios, i.e. 2.5, due to the excess substrate's inhibition of microbial enzymes (Parawira et al., 2004).

4.7 Carbohydrate content and profile

Enzyme loadings were chosen based on previous research that showed an enzyme cocktail of cellulase, pectinase, xylanase, and hemicellulase (0.4% w/w each) displayed the greatest increase in reducing sugar content (data not shown).

The control olive cake had a similar amount of glucose and xylose/galactose (each ~5.3 mg/mL, Table 4.4). After enzymatic pretreatment, concentrations of glucose in the EDOC (9.9 mg/mL) and the destoned enzymatically pretreated olive cake (DEDOC, 10.4 mg/mL) significantly increased due to cellulase activity. Cellulase enzymes depolymerize cellulose before hydrolyzing it into cellodextrins. Cellobiases further hydrolyzes cellodextrin into glucose, the monomeric form of cellulose.

Xylose/galactose content stayed unchanged. Glucuronoxylans, which can be degraded into xylose/galactose are commonly found in the lignified stone in the olive pulp (Coimbra et al., 1995; Coimbra et al., 1994). Due to the crystalline structure of the lignin, the hemicellulases and xylanses were unable to convert those xylans into xylose under the conditions used in the experiment.

Arabinose was not detected in the DOC and DDOC; however, 2.5 mg/mL was found in both EDOC and DEDOC. Arabinofuranosyl is a common sidechain in hemicellulose that hinders xylanase access to the xylan backbone of the hemicellulose and cellulase access to cellulose. Arabinases that are present in hemicellulase, α-L-

arabinofuranoside and α-L-arabinase, to hydrolyze these sidechains, resulting in the production of arabinose. Due to the lignin, which hinders enzymatic access to hemicellulose, none of the arabinose obtained was expected to be from hemicellulose. Instead, the arabinose was obtained from the hydrolysis of the arabinan pectins ((1→5)-α-L arabinan chains) that are unique to olives. Most of the insoluble pectin in olive cake is composed of arabinan chains. However, there is a small fraction of insoluble pectin (2.6-9.7%) that is rich in galacturonic acid. This is because as olives ripen, a large fraction of the galacturonic pectin becomes water soluble (Vierhuis et al., 2000). During the centrifugation step of oil extraction, the water-soluble galacturonic pectin partitions into the olive mill wastewater. In fact, this pectin makes up 94% of the soluble fiber in OMWW (Dermeche et al., 2013). Arabinan side chains linked to rhamnose are interspersed in the galacturonic acid chains that make up the backbone of the insoluble pectin in the olive cake (Tahkur et al., 1997). Therefore, the use of pectinase in olive cake increases arabinase access to these side chains by hydrolyzing the galacturonic acid chains.

After the samples were prepared for anaerobic digestion (day 0), an expected decrease of total sugar content was observed due to the dilution performed. After 15 days of digestion, no more monomers were present. These results were expected due to the immediate consumption of glucose. The rate of hydrolysis of cellulose (0.6 d^{-1}) is lower than the rate of fermentation of its monomeric product, glucose (3.8 d^{-1}) meaning that accumulation of glucose was non-existent (Gujer and Zehnder, 1983; Fernández et al., 2011). The hydrolysis of hemicellulose and the fermentation of xylose and arabinose followed similar mechanisms than the one for glucose (Qureshi, 2009). Similarly, the

total carbohydrates remaining in the digestate supernatant at the end of a 22 day digestion period of a two-phases olive cake for methane production was found to be negligible (0.025 – 0.050 mg/mL) (Serrano et al, 2017).

Table 4.4. Carbohydrate profile of olive cake supernatants and digestates at Day 0, 15, and 30

Substrate		Glucose (mg/mL)	Xylose/Galactose (mg/mL)	Arabinose (mg/mL)	Total (mg/mL)
DOC[1]	Pre-digestion	5.34 ± 1.50^{c}	5.38 ± 1.49^{c}	n.d.	10.72 ± 3.00^{c}
Digestate	Day 0	0.18 ± 0.08^{a}	0.17 ± 0.08^{a}	n.d.	0.35 ± 0.16^{a}
	Day 15	n.d.	n.d.	n.d.	n.d.
	Day 30	n.d.	n.d.	n.d.	n.d.
DDOC[2]	Pre-digestion	6.80 ± 2.48^{cd}	6.49 ± 2.33^{c}	n.d.	13.29 ± 4.80^{c}
Digestate	Day 0	0.44 ± 0.14^{ab}	0.68 ± 0.19^{b}	n.d.	1.12 ± 0.33^{b}
	Day 15	n.d.	n.d.	n.d.	n.d.
	Day 30	n.d.	n.d.	n.d.	n.d.
EDOC[3]	Pre-digestion	9.88 ± 0.48^{d}	6.61 ± 1.90^{c}	2.45 ± 0.21^{b}	18.94 ± 2.35^{c}
Digestate	Day 0	0.61 ± 0.15^{b}	0.44 ± 0.10^{ab}	0.18 ± 0.04^{a}	1.23 ± 0.29^{b}
	Day 15	n.d.	n.d.	n.d.	n.d.
	Day 30	n.d.	n.d.	n.d.	n.d.
DEDOC[4]	Pre-digestion	10.35 ± 0.44^{d}	6.23 ± 0.52^{c}	2.53 ± 0.16^{b}	19.11 ± 0.95^{c}
Digestate	Day 0	0.63 ± 0.02^{b}	0.47 ± 0.00^{ab}	0.13 ± 0.02^{a}	1.23 ± 0.01^{b}
	Day 15	n.d.	n.d.	n.d.	n.d.
	Day 30	n.d.	n.d.	n.d.	n.d.

Different letters within a group of compounds indicates significant differences. 1. Digestate of diluted olive cake, control; 2. Digestate of destoned diluted olive cake; 3. Digestate of enzymatically pretreated diluted olive cake; 4. Digestate of destoned and enzymatically pretreated diluted olive cake

Other possible substrates converted during digestion were lipids and proteins, which produce more methane per g of VS (Wilkie, 2008). The Kjeldahl method was used to estimate protein content based off of total nitrogen. Since the main products of anaerobic digestion are methane and carbon dioxide, the nitrogen content in the anaerobic digester should remain unchanged (Makádi et al., 2012). Accordingly, the amount of proteins digested is unable to be calculated from the current data. The use of SDS-PAGE

gel to visualize the peptide profile of the olive cake before and after digestion should help verify protein digestion. About 65% of the fat was shown to be digested.

4.8 Valorization potential of the digestate

Undigested olive cake has a gross heat of combustion up to 21 kJ/g TS, which is comparable to coconut fiber (20 kJ/g TS), grape pomace (20.3 kJ/g TS), and slightly lower than soft coal (23 kJ/g TS) (Domalski et al., 1986; Ferrer et al., 2018). Because of this high caloric value, olive cakes are currently burned for energy in Mediterranean countries. In our study, the digestates recovered after anaerobic digestion of the olive cakes that produced the highest amounts of methane, the destoned diluted olive cake (DDOC) and the enzymatically pretreated olive cake (EDOC), were further analyzed to determine their possible uses (Table 4.5.). The olive cake digestates had a low heating combustion values of 3.3-3.5 kJ/g TS, limiting their value for heat recovery by incineration. This low heat value was attributed to the dilution of the sample necessary for anaerobic digestion and subsequent biomethane production.

The Kjeldahl nitrogen contents of olive cake digestates (DDOC and EDOC) were 3.7% (db), which were higher than the undigested olive cake (1.4%, db) and slightly lower than cow manure's digestate (4.3%, db) (Table 4.5) (Makádi et al., 2012). Increased nitrogen content in the digested olive cakes could increase their value as soil amendment. Indeed, as nitrogen is the main limiting factor for plant growth, incorporating olive cake digestate as a soil amendment would lower the need for artificial fertilizers created using fossil fuels. Furthermore, under the US Food Safety Modernization Act, olive cake digestate could be an attractive fertilizer for organic farmers when digested with an inoculum that has no human nor animal wastes (Food and

Drug Administration, 2013). Another advantage of using olive cake digestate as a soil amendment instead of directly applying olive cake is its low phenolic load (~0.020 mg GAE/g), significantly reducing its phytotoxic effect.

Van Soest sequential fiber analysis showed the digestates were composed of about 17% (db) hemicellulose and 12% (db) lignin. A majority of the insoluble carbohydrates came from the inoculum, digested wastewater sludge, resulting in high percentages of hemicellulose and lignin in digestates (Table 4.5).

At the same time, none of hemicellulose from the olive cake was converted into soluble carbohydrates during the anaerobic digestion process. This is because a large portion of hemicellulose in olive cake is bound by lignin and the lignin must be degraded before enzymes can access this hemicellulose (Coimbra et al., 1994). Destoning removes a majority of the stones, which are composed of lignin. However, destoning does not remove other sources of lignin in the olive cake (heavily lignified olive pulp that was close to the olive stones) or compounds that are similar to lignin (polysaccharide-protein-phenolic complexes). Therefore, there will be residual lignin that has hemicellulose trapped inside of it, which microbial enzymes are unable to convert to soluble carbohydrates. On the other hand, 42% of the cellulose, a major component of olive pulp, was converted into soluble carbohydrates during the 30 day anaerobic digestion. High fiber digestates have been sold as a peat substitute for potting mixes (Pelaez-Samaniego et al., 2017).

4.9 Scale-up biochemical methane potential (BMP) assay

Small-scale batch anaerobic digesters are known to possibly overestimate the amount of methane produced (Holliger et al., 2017). Therefore, the digestion of destoned

(DDOC) and enzymatically pretreated (EDOC) olive cakes were scaled up to a working volume of 1 L (2.3 L glass vessels) in order to determine the impact of the scale-up on the specific methane yields (SMY) obtained in the small-scale (100 mL) experiment.

Table 4.5. Characteristics of digestate after 30 days of anaerobic digestion

Characteristics	Inoculum	DDOC[1]	EDOC[2]
Total Solids (g/kg)	21.35 ± 0.03^{a}	26.55 ± 0.05^{b}	29.40 ± 0.45^{b}
Kjeldahl Nitrogen (%TS)	3.99 ± 0.05^{a}	3.69 ± 0.07^{b}	3.66 ± 0.01^{b}
Crude Fat (%TS)	3.18 ± 0.19^{a}	3.16 ± 0.31^{a}	3.18 ± 0.33^{a}
Volatile Solids (%TS)	59.08 ± 0.62^{a}	66.94 ± 0.43^{ab}	73.74 ± 7.78^{b}
Ash (%TS)	40.92 ± 0.62^{b}	33.05 ± 0.43^{ab}	26.25 ± 7.78^{a}
pH	8.59 ± 0.02^{c}	7.52 ± 0.02^{a}	7.73 ± 0.03^{b}
Hemicellulose (%TS)	15.59 ± 1.19^{a}	16.68 ± 1.20^{b}	17.32 ± 1.55^{b}
Cellulose (%TS)	2.02 ± 0.79^{a}	4.64 ± 0.95^{b}	4.62 ± 0.51^{b}
Lignin (%TS)	10.32 ± 0.38^{a}	11.68 ± 0.71^{b}	11.86 ± 0.61^{b}
Total Phenolics (mg GAE/g)	0.017 ± 0.001^{a}	0.026 ± 0.001^{a}	0.019 ± 0.003^{a}
Gross Heat of Combustion (kJ/g TS)	3.11 ± 0.04^{a}	3.31 ± 0.17^{a}	3.65 ± 0.43^{a}

Different letters for each characteristic indicates a significant difference. 1. Destoned diluted olive cake; 2. Enzymatically pretreated diluted olive cake; 3. Acid detergent lignin; 4. mg GAE/g = mg gallic acid equivalent/g of extract; TS = Total solids (dry matter)

The 1 L DDOC digester produced 276 mL CH_4/g VS at the end of the 30 day digestion period, which was 94% of the SMY of the 100 mL digesters. Alternatively, the EDOC only produced 98 mL CH_4/g VS, 33% of the SMY of the corresponding 100 mL digester (Fig 4.7). Following the same trend, from day 16 onwards, the biogas produced from DDOC was composed of 60-70% methane, whereas the EDOC was composed of a maximum of 56% (Fig 4.8).

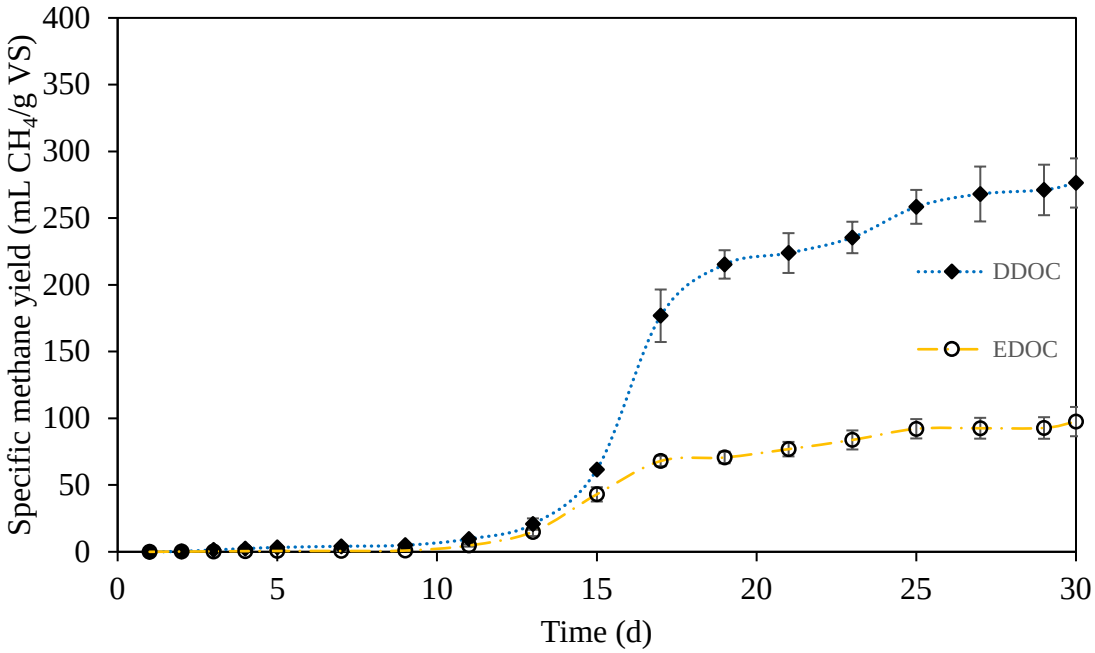

Fig 4.7. Effect on enzyme and destoning on specific methane yield (SMY) at a 1 L scale

Destoned diluted olive cake (DDOC); enzymatically pretreated diluted olive cake (EDOC)

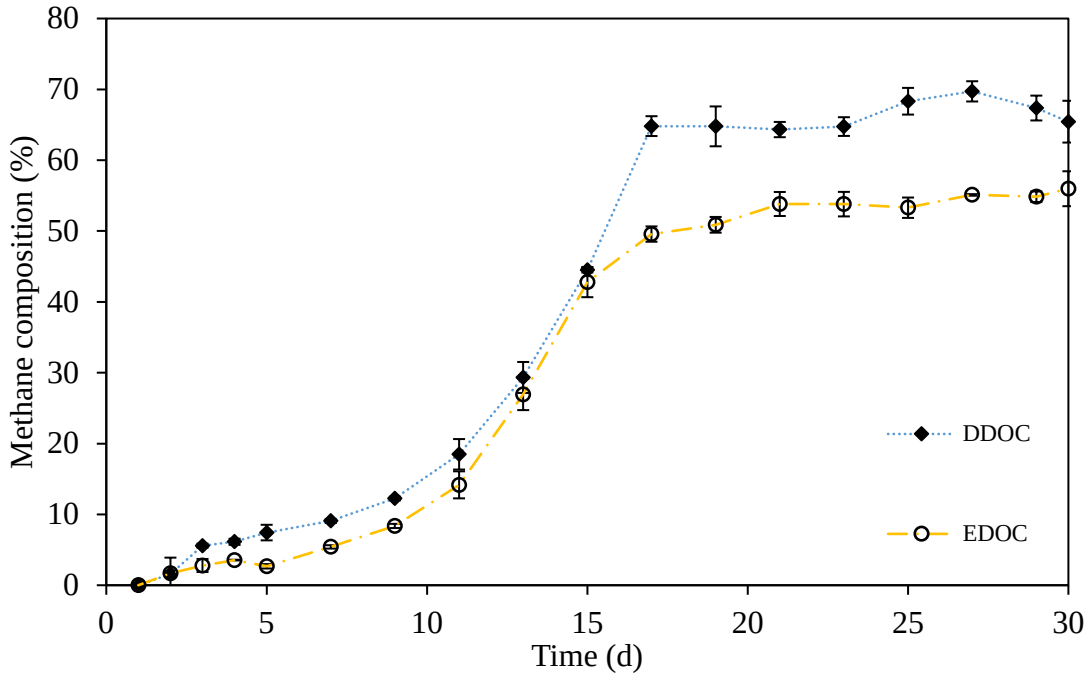

Fig 4.8. Methane composition of biogas produced from the anaerobic digestion of pretreated olive cakes at 1 L scale

Destoned diluted olive cake (DDOC); enzymatically pretreated diluted olive cake (EDOC)

The low biomethane yield of the EDOC is likely due to the high pH of the digester (7.7), which almost exceeded the limit of anaerobic digestion (Fig 4.9). The same inoculum was used for both the 100 mL and the 1 L anaerobic digesters to decrease variation. During the month between the small-scale and 1 L experiments, the pH of the inoculum had increased from 7.9 to 8.6. A pH that is higher than the pH limit of anaerobic digestion can shift the ammonia-ammonium equilibrium, leading to the conversion of ammonium to ammonia in the digester. Ammonia is not only inhibitory to anaerobic microorganisms, but also produces malodors, reduces methane production, and results in a low percentage of methane in the biogas (Strik et al., 2006). For future studies, the pH will need to be adjusted so that it remains within the recommended limits.

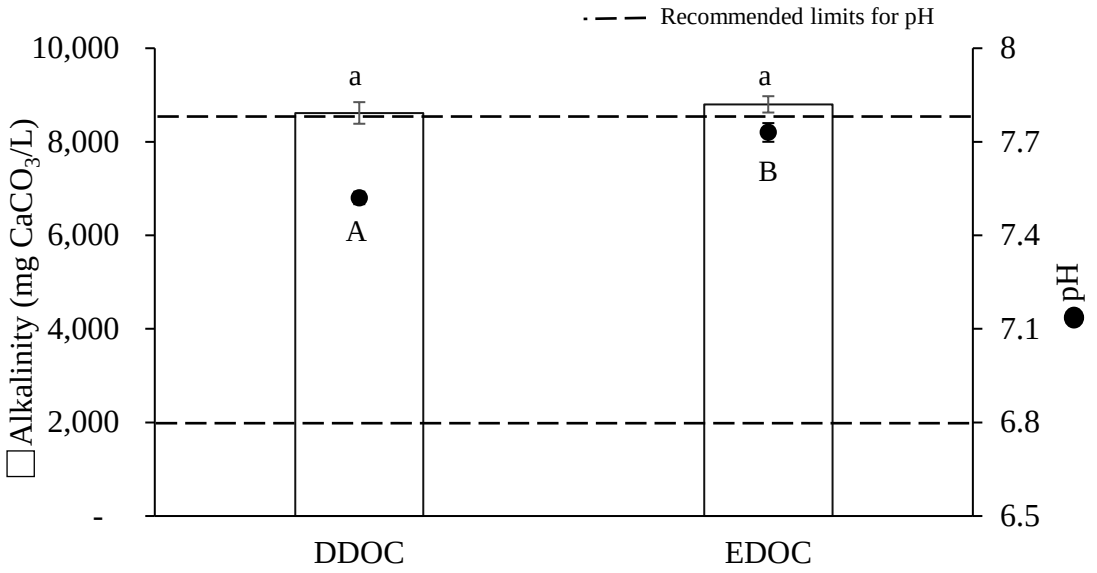

Fig 4.9. Alkalinity and pH after 30 days of anaerobic digestion at 1L scale
Destoned diluted olive cake (DDOC); Enzymatically pretreated diluted olive cake (EDOC)

4.10 Ethanol production

There are two processes usually employed in the aerobic fermentation of lignocellulosic substrates such as olive cake: simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF). SSF can help reduce the formation of inhibitory end products, the need for separate reactors, and the chance of contamination. Nonetheless, ethanol production is conventionally done with a SHF process, as there is often a difference between optimal enzyme and fermentation conditions (Olofsson et al., 2008). The optimal temperature for the growth of *Saccharomyces cerevisiae* is around 30 °C, while it is around 55 °C for carbohydrase enzymes. If the SSF method is chosen, a temperature that either runs the risk of stressing the yeast or one that results in a sub-optimal enzymatic hydrolysis rate must be selected. A common temperature range for SFF is 35 – 38 °C (Olofsson et al., 2008).
In this experiment, the fermentation of the enzymatically pretreated olive cake (EDOC) as well as the destoned and enzymatically pretreated olive cake (DEDOC) have undergone the SHF process.

The olive cakes that were not submitted to enzymatic pretreatments (DOC and DDOC) did not produce any ethanol, even though all the glucose present was consumed (Table 4.5). The inhibition of the production of ethanol by *Saccharomyces cerevisiae* occurs when a high concentration of polyphenols is present (3.8-8.6 mg GAE/mL) (Fleming et al., 1973, Zanichelli et al., 2007). The olive cake used for the fermentation had a total phenol content of about 1.0 mg GAE/g, so inhibition due to phenolic compounds was not expected.

The likely cause of the inhibition that occurred in the non-enzyme pretreated samples was the unexpected production of acetic acid, which strongly inhibits yeast growth and ethanol production. The undissociated form of the molecule diffuses easily across the phospholipid section of the plasma membrane and then dissociates within the yeast cell, disrupting the intracellular pH (Narendranath et al., 2001). About 7.5 mg/mL of acetic acid was reported to reduce *S. cerevisiae* biomass yield by 80%, but only 0.5 mg/mL was necessary to show an inhibitory effect (Maiorella et al., 1983; Narendranath et al., 2001). The absence of ethanol after the control (DOC) and DDOC were fermented is likely the result of low glucose levels (0.23 and 0.59 mg/mL, respectively) compounded by the presence of acetic acid during the fermentation (0.17 and 0.62 mg/mL, respectively) (Table 4.5). The temperature known to produce a stress response in *S. cerevisiae* and therefore promote the production of acetic acid, 42 °C, was never reached when activating the yeast or during the fermentation (Woo et al., 2014). Lactrol was used to eliminate possible microbial contamination. Results have shown that at the concentration of Lactrol used for this experiment (2 ppm), no bacterial cells could be found when the fermentation ended (Muhammad et al., 2011). However, the active antibiotic in Lactrol is virginiaycin, which cannot penetrate the cell membrane of gram-negative bacteria such as acetic acid bacteria (AAB) (Nott et al., 2009).

Unlike the DOC and DDOC, ~1.70 mg/mL of ethanol was detected at time 0 h as well as 72 h for the EDOC (Table 4.6.). Different species of yeasts that can produce ethanol have been isolated from two-phase olive mill waste (2POMW) (Giannoutsou et al., 2004; Abu Tayeh et al., 2014). Considering that olive cake has a lower polyphenol

concentration than 2POMW, it would not be unexpected to find indigenous yeast utilizing the substrate to produce ethanol during the enzymatic pretreatment.

The destoned and enzymatically pretreated olive cake (DEDOC) produced 1.27 mg/mL of ethanol, which was 53% of the theoretical amount based on the glucose consumed. Haagensen et al. (2009) obtained a similar yield, 67% of the theoretical yield of ethanol when fermenting 2POMW using a SHF method. Using an SSF procedure produced 86% of the theoretical yield. The lack of ethanol produced by the SHF method in Haagensen et al.'s experiment was similarly attributed to microbial contamination.

The use of microorganisms other than *S. Cerevisiae* to ferment olive cake has also been investigated. *Thermoanaerobacter ethanolicus* is able to break down complex carbohydrates, supposedly eliminating the need for enzymatic hydrolysis of the olive cake. But, the organism required glucose supplementation to overcome any inhibitory effects of the olive cake. Using *T. ethanolicus* to ferment 50 g TS/L of olive cake produced 0.65 mg/mL of ethanol, 20% of the theoretical yield. In addition, 0.22 mg/mL of acetic acid was produced during the fermentation (Jurado et al., 2010). The use of *E. coli* FBR5 in fermenting 135 g TS/L of dilute-acid hydrolyzed olive cake (1.75% w/v H_2SO_4 at 160 °C for 10 min) by SSF was more successful. At the end of the 3 day fermentation period, 8.1 mg/mL of ethanol, 92% of the theoretical yield, was produced (El Asli and Qatibi, 2009). However, when the temperature of the dilute acid pretreatment was raised by 20 °C to 180 °C, no ethanol was produced from the subsequent fermentation. One of the pitfalls of the pretreatments listed above are costs as well as environmental impact. Moreover, as previously discussed, pretreatments like wet

oxidization and dilute acid create inhibitory products that reduce or inhibit the final ethanol yield (El Asli and Qatibi, 2009; Haagensen et al., 2009).

Compared to other agricultural resides, the bioethanol potential of olive mill wastes (olive cake and 2POMW) is low (Table 4.7). High total solids loadings (20 – 30% TS) are required for olive mill wastes to produce as much ethanol as coffee husks (11% TS) and barley straw (unreported TS loading). If olive cake is to become a substrate for ethanol production, the first major hurdle is the control of endogenous microorganisms that produce organic acids such as lactic acid or acetic acid, which inhibit or reduce ethanol production. Two types of research have flourished from this need. The first type of research is the use of yeasts indigenous to OMW to produce ethanol (Abu Tayeh et al., 2014). The second is to increase the tolerance of microorganisms to inhibitors such as acetic acid through metabolic evolution (Martínez-Patiño et al., 2015)

Table 4.6. Concentration of carbohydrate and fermentation products of olive cake batch fermentation supernatant at 0 and 72 h

Substrate	Time (h)	Glucose (mg/mL)	Xylose/ Galactose (mg/mL)	Arabinose (mg/mL)	Lactic Acid (mg/mL)	Acetic Acid (mg/mL)	Ethanol (mg/mL)
DOC	0	0.25 ± 0.06^{a}	0.72 ± 0.24^{cd}	n.d.	0.10 ± 0.10^{a}	0.17 ± 0.17^{b}	n.d.
	72	n.d.	0.38 ± 0.06^{d}	n.d.	0.10 ± 0.10^{a}	n.d.	n.d.
DDOC	0	0.59 ± 0.14^{a}	2.25 ± 0.53^{ab}	n.d.	0.35 ± 0.30^{a}	0.67 ± 0.27^{b}	n.d.
	72	n.d.	1.78 ± 0.72^{b}	n.d.	0.34 ± 0.25^{a}	n.d.	n.d.
EDOC	0	1.78 ± 0.37^{b}	1.39 ± 0.04^{bcd}	0.46 ± 0.00^{c}	n.d.	0.19 ± 0.19^{a}	1.71 ± 0.07^{b}
	72	n.d.	1.26 ± 0.03^{bcd}	0.50 ± 0.01^{c}	n.d.	n.d.	1.69 ± 0.07^{b}
DEDOC	0	4.68 ± 1.57^{c}	3.15 ± 1.00^{a}	1.26 ± 0.11^{a}	0.11 ± 0.15^{a}	0.71 ± 0.50^{ab}	0.80 ± 0.24^{c}
	72	n.d.	1.70 ± 0.64^{abc}	0.77 ± 0.08^{b}	n.d.	n.d.	2.07 ± 0.32^{a}

Different letters indicate significant differences between substrates for one compound; n.d. = not detected; diluted olive cake (DOC, control); destoned diluted olive cake (DDOC); enzymatically pretreated olive cake (EDOC); destoned and enzymatically pretreated olive cake (DEDOC)

Table 4.7. Comparison of ethanol yields obtained during separate hydrolysis and fermentation

Substrate	Solid Loading (g TS/L)	Microorganism	Ethanol (mg/mL)	Theoretical Yield (%)	Reference
2POMW[1]	100	*S.cerevisiae*	3.7	97	Georgieva and Ahring, 2007
2POMW[1]	300	*S.cerevisiae*	11.2	100	Georgieva and Ahring, 2007
Banana stems	151	*S.cerevisiae* (Ethanol Red)	42.0 ± 0.9	74.3	Guerrero et al., 2018
Barley Straw		*S.cerevisiae*	10	84 - 90	Belkacemi et al., 2002
Coffee Husk	110	*S.cerevisiae* (Fleischman)	11.5	62.7	Gouvea et al., 2009
Corn Stalk		*S.cerevisiae*	5.0	84 - 90	Belkacemi et al., 2002
DEDOC[2]	50	*S.cerevisiae* (Ethanol Red)	1.3 ± 0.3	53	
Wheat straw	86	*E. coli* (FBR5)	21.9 ± 0.3	97.3	Saha et al., 2011

1. Two-phase olive mill waste; 2. Destoned and enzymatically pretreated olive cake;

CHAPTER 5 – CONCLUSION

The purpose of this research was to determine the value of olive cake as a substrate for biofuel (bioethanol and biomethane) production and whether or not its value could be increased if environmentally friendly pretreatments such as destoning and enzymes were applied. It was hypothesized that destoning and enzymatically pretreating olive cake would increase its biomethane and bioethanol yields.

Removing the olive stones with a horizontal screw press (HSP) proved to be a better destoning method than destoning with a centrifuge. While both methods removed the same amount of stones (~95%) from enzymatically pretreated olive cake, a larger portion of the digestible total solids (pulp) remained separated from the stone fraction when a HSP was used. The purchase of a HSP is an additional capital cost for olive mill owners. However, this cost many be offset if olive mill wastewater can be used to dilute olive cake so it is fit for destoning. More investigation of the economics of destoning by screening is necessary. Moreover, enzymatically pretreating the olive cake before destoning allows for the recovery of stones that do not have attached insoluble fibers. The stones may be purposed as construction materials, filler for plastics, or activated carbon rather than just being disposed in a landfill.

Both destoning by HSP as well as enzymatic pretreatment increased olive cake's suitability for producing biomethane. Under similar conditions, the destoned and enzymatically pretreated olive cakes produced 24 and 17% more methane, respectively, than corn stover and vinegar residue, which are agricultural residues commonly considered for anaerobic digestion. The remaining digestate was found to have value as a soil amendment due to its high nitrogen content and low polyphenol concentration.

Therefore, it has been shown that anaerobic digestion is a promising pathway for the valorization of olive cake. Further research into this topic should include whether or not the specific methane yields obtained in this experiment can be reproduced when the anaerobic digestion is scaled up to a semi-continuous and continuous process (Tekin and Dalgıç, 2000; Grosser, 2018).

The fermentation of olive cake was disrupted due to microbial contamination that either inhibited or reduced the amount of ethanol produced. The literature has also shown that it is difficult to produce ethanol with olive cake due to the inhibitory compounds that are either indigenous to the olive cake or are produced when the olive cake is pretreated. If the industrial fermentation of olive cake is to be pursued, more research on the optimization of pretreatment processes to control the production of inhibitory compounds or different ways to avoid microbial contamination are necessary.

It was concluded that sustainable pretreatments can help increase the value of olive cake as feedstock for second generation biofuel production, diverting this waste material from landfill, lowering GHG emissions and proving value for olive producers and processors in California.

BIBLIOGRAPHY

Aasberg-Petersen, K., Nielsen, C.S., Dybkj, I., 2008. Large scale methanol production from natural gas. Haldor Topsoe, Ravnholm, pp 1–14.

Abdelhadi, S.O., Dosoretz, C.G., Rytwo, G., Gerchman, Y., Azaizeh, H., 2017. Production of biochar from olive mill solid waste for heavy metal removal. Bioresour. Technol. 244, 759–767.

Abu Tayeh, H., Najami, N., Dosoretz, C., Tafesh, A., Azaizeh, H., 2014. Potential of bioethanol production from olive mill solid wastes. Bioresour. Technol. 152, 24–30.

Abu-Zreig, M., Al-Widyan, M., 2002. Influence of olive mills solid waste on soil hydraulic properties. Commun. Soil Sci. Plant Analysis 33, 505–517.

Angelidaki, I., Ahring, B.K., 1992. Effects of free long-chain fatty acids on thermophilic anaerobic digestion. App. Microbiology Biotechnol. 37, 808–812.

Angelidaki, I., Alves, M., Bolzonella, D., Borzacconi, L., Campos, J.L., Guwy, A.J., Kalyuzhnyi, S., Jenicek, P., van Lier, J.B., 2009. Defining the biomethane potential (BMP) of solid organic wastes and energy crops: a proposed protocol for batch assays. Water Sci. Technol. 59, 927–934.

Alzate, M.E., Muñoz, R., Rogalla, F., Fdz-Polanco, F., Pérez-Elvira, S.I., 2012. Biochemical methane potential of microalgae: influence of substrate to inoculum ratio, biomass concentration and pretreatment. Bioresour. Technol. 123, 488–494.

Akassou, M., Kaanane, A., Crolla, A., Kinsley, C., 2010. Statistical modelling of the impact of some polyphenols on the efficiency of anaerobic digestion and the co-digestion of the wine distillery wastewater with dairy cattle manure and cheese whey. Water Sci. Technol. 62, 475–483.

Amirante, P., Clodoveo, M.L., Dugo, G., Leone, A., Tamborrino, A., 2006. Advance technology in virgin olive oil production from traditional and de-stoned pastes: influence of the introduction of a heat exchanger on oil quality. Food Chem. 98, 797–805.

Andre, R., Pinto, F., Franco, C., Dias, M., Gulyurtlu, I., Matos, M., Cabrita, I., 2005. Fluidised bed co-gasification of coal and olive oil industry wastes. Fuel 84, 1635–1644.

Armesto, L., Bahillo, A., Cabanillas, A., Veijonen, K., Otero, J., Plumed, A., Salvador, L., 2003. Co-combustion of coal and olive oil industry residues in fluidised bed. Fuel 82, 993-1000.

AOAC., 2005. Official methods of analysis of AOAC International. eighteenth ed. Association of Analytical Chemistry, Maryland.

AOAC., 1990. Official methods of analysis of AOAC International. fifteenth ed. Assoc. Analytical Chemistry, Maryland.

APHA., 1985. Standard methods for the examination of water and wastewater, sixteenth ed. Am. Public Health Assoc., Washington DC.

Atimtay, A.T., Topal, H., 2004. Co-combustion of olive cake with lignite coal in a circulating fluidized bed. Fuel 83, 859–867.

Azbar, N., Bayram, A., Filibeli, A., Muezzinoglu, A., Sengul, F., Ozer, A., 2004. A review of waste management options in olive oil production. Critical Rev. Environ. Sci. Technol. 34, 209–247.

Baeta-hall, L., 2005. Bio-degradation of olive oil husks in composting aerated piles. Bioresour. Technol. 96, 69–78.

Ballesteros, I., Oliva, J.M., Negro, M.J., Manzanares, P., Ballesteros, M., 2002. Ethanol production from olive oil extraction residue pretreated with hot water. App. Biochem. Biotechnol. 98–100, 717–732.

Bambalov, G., Israilides, C., Tanchev, S., 1989. Alcohol fermentation in olive oil extraction effluents. Biological Wastes 27, 71–75.

Bansal, P., Hall, M., Realff, M.J., Lee, J.H., Bommarius, A.S., 2009. Modeling cellulase kinetics on lignocellulosic substrates. Biotechnol. Adv. 27, 833–848.

Barreca, F., Fichera, C.R., 2013. Use of olive stone as an additive in cement lime mortar to improve thermal insulation. Energy and Buildings 62, 507–513.

Bateman, D.F., Millar, R.L., 1966. Pectic enzymes in tissue degradation. Annual Rev. Phytopathol. 4, 119–144.

Belkacemi, K., Turcotte, G., Savoie, P., 2002. Aqueous/steam-fractionated agricultural residues as substrates for ethanol production. Indust. Eng. Chem. Res. 41, 173–179.

Berbel, J., Posadillo, A., 2018. Review and analysis of alternatives for the valorisation of agro-industrial olive oil by-products. Sustain. 10, 1–9.

Binod, P., Janu, K.U., Sindhu, R., Pandey, A., 2011. Hydrolysis of lignocellulosic biomass for bioethanol production, in: Pandey, A., Larroche, C., Ricke, S., Dussap, C.G., Gnansounou, E. (Eds.), Biofuels. Elsevier, Oxford, pp. 229–250.

Biswas, R., Uellendahl, H., Ahring, B.K., 2015. Wet explosion: a universal and efficient pretreatment process for lignocellulosic biorefineries. Bioeng. Res. 8, 1101–1116.

Blanquez, P., Caminal, G., Sarra, M., Vicent, M.T., Gabarrell, X., 2002. Olive oil mill waste waters decoloration and detoxification in a bioreactor by the white rot fungus *Phanerochaete flavido-alba*. Biotechnol. Progress 18, 660–662.

Borja, R., Raposo, F., Rincón, B., 2006. Treatment technologies of liquid and solid wastes from two-phase olive oil mills. Grasas y Aceites 57, 32–64.

Brummer, V., Skryja, P., Jurena, T., Hlavacek, V., Stehlik, P., 2014. Suitable technological conditions for enzymatic hydrolysis of waste paper by Novozymes® enzymes NS50013 and NS50010. App. Biochem. Biotechnol. 174, 1299–1308.

CalRecycle, 2004. Compost quality standards. Available from <http://www.calrecycle.ca.gov/organics/Products/Quality/CQStandards.htm> (accessed on 5.10.18).

CalRecycle, 2015. Landfill tipping fees in California. Available from <http://www.calrecycle.ca.gov/Publications/Documents/1520/20151520.pdf> (accessed 3.29.18).

Cara, C., Moya, M., Ballesteros, I., Negro, M.J., González, A., Ruiz, E., 2007. Influence of solid loading on enzymatic hydrolysis of steam exploded or liquid hot water pretreated olive tree biomass. Process Biochem. 42, 1003–1009.

Cardoso, S.M., Coimbra, M.A., Lopes da Silva, J., 2003. Calcium-mediated gelation of an olive pomace pectic extract. Carbohydr. Polymers 52, 125–133.

Chandra, R.P., Bura, R., Mabee, W.E., Berlin, A., Pan, X., Saddler, J.N., 2007. Substrate pretreatment: the key to effective enzymatic hydrolysis of lignocellulosics, in: Olsson, L. (Ed.), Biofuels. Springer Berlin Heidelberg, Berlin, pp. 67–93.

Che, F., Sarantopoulos, I., Tsoutsos, T., Gekas, V., 2012. Exploring a promising feedstock for biodiesel production in Mediterranean countries: a study on free fatty acid esterification of olive pomace oil. Biomass and Bioeng. 36, 427–431.

Chen, S., Zhang, J., Wang, X., 2015. Effects of alkalinity sources on the stability of anaerobic digestion from food waste. Waste Management & Res. 33, 1033–1040.

Chiofalo, B., Liotta, L., Zumbo, A., Chiofalo, V., 2004. Administration of olive cake for ewe feeding: effect on milk yield and composition. Small Rumin. Res. 55, 169–176.

Chynoweth, D.P., Turick, C.E., Owens, J.M., Jerger, D.E., Peck, M.W., 1993. Biochemical methane potential of biomass and waste feedstocks. Biomass and Bioeng. 5, 95–111.

Cicerale, S., Conlan, X.A., Sinclair, A.J., Keast, R.S.J., 2008. Chemistry and health of olive oil phenolics. Critical Rev. Food Sci. Nutr. 49, 218–236.

Coimbra, M., 1994. Isolation and characterization of cell wall polymers from olive pulp (*Olea europaea L.*). Carbohydr. Res. 252, 245–262.

Coimbra, M., Waldron, K., Selvendran, R. 1995. Isolation and characterization of cell wall polymers from the heavily lignified tissues of olive (*Olea europaea*) seed hull. Carbohydr. Polymer. 27, 285–294.

Coimbra, M.A., Cardoso, S.M., Lopes-da-Silva, J.A., 2010. Olive pomace, a source for valuable arabinan-rich pectic polysaccharides, in: Rauter, A.P., Vogel, P., Queneau, Y. (Eds.), Carbohydrates in sustainable development I. Springer, Heidelberg, pp. 129–141.

Dal Bosco, A., Castellini, C., Cardinali, R., Mourvaki, E., Moscati, L., Battistacci, L., Servili, M., Taticchi, A., 2010. Olive cake dietary supplementation in rabbit: immune and oxidative status. Italian J. Animal Sci. 6, 761–763.

Davis, K., Rover, M., Brown, R., Bai, X., Wen, Z., Jarboe, L., 2016. Recovery and utilization of lignin monomers as part of the biorefinery approach. Energies 9, 808 - 836.

DellaGreca, M., Monaco, P., Pinto, G., Pollio, A., Previtera, L., Temussi, F., 2001. Phytotoxicity of low-molecular-weight phenols from olive mill waste waters. Bulletin of Environ. Contamination and Toxicology 67, 352–359.

de Almeida Antunes Ferraz, J.L., Souza, L.O., Soares, G.A., Coutinho, J.P., de Oliveira, J.R., Aguiar-Oliveira, E., Franco, M., 2018. Enzymatic saccharification of lignocellulosic residues using cellulolytic enzyme extract produced by *Penicillium roqueforti* ATCC 10110 cultivated on residue of yellow mombin fruit. Bioresour. Technol. 248, 214–220.

de Jong, E., Jungmeier, G., 2015. Biorefinery concepts in comparison to petrochemical refineries. in: Pandey, A., Hofer, R., Taherzadeh, M., Nampoothiri, K. M., Larroche, C., (Eds.), Industrial biorefineries & white biotechnology, Elsevier, Amsterdam, pp 3–33.

Dhar, B.R., Youssef, E., Nakhla, G., Ray, M.B., 2011. Pretreatment of municipal waste activated sludge for volatile sulfur compounds control in anaerobic digestion. Bioresour. Technol. 102, 3776–3782.

Di Giovacchino, L., Costantini, N., Ferrante, M.L., Serraiocco, A., 2002. Influence of malaxation time of olive paste on oil extraction yields and chemical and organoleptic characteristics of virgin olive oil obtained by a centrifugal decanter at water saving. Grasas y Aceites 53, 179–186.

Domalski, E.S., Jobe, Jr, T.L., Milne, T.A., 1986. Thermodynamic data for biomass conversion and waste incineration. Available from <https://www.osti.gov/biblio/7038865/> (accessed 3.29.18).

Edeseyi, M.E., Kaita, A.Y., Harun, R., Danquah, M.K., Acquah, C., Sia, J.K.M., 2015. Rethinking sustainable biofuel marketing to titivate commercial interests. Renew. Sustain. Energy Rev. 52, 781–792.

EIA., 2017. State energy data system: 1960-2015. Available from <https://www.eia.gov/state/seds/seds-data-complete.php?sid=CA> (accessed 5.10.18)

EPA., 2017. Understanding global warming potentials. Available from <https://www.epa.gov/ghgemissions/understanding-global-warming-potentials> (accessed 5.10.18)

El Asli, A., Qatibi, A.-I., 2009. Ethanol production from olive cake biomass substrate. Biotechnol. Bioprocess Eng. 14, 118–122.

ElMekawy, A., Diels, L., Bertin, L., De Wever, H., Pant, D., 2014. Potential biovalorization techniques for olive mill biorefinery wastewater. Biofuels, Bioproducts and Biorefining 8, 283–293.

Ergüder, T., Güven, E., Demirer, G., 2000. Anaerobic treatment of olive mill wastes in batch reactors. Process Biochem. 36, 243–248.

Eskicioglu, C., Ghorbani, M., 2011. Effect of inoculum/substrate ratio on mesophilic anaerobic digestion of bioethanol plant whole stillage in batch mode. Process Biochem. 46, 1682–1687.

Felizón, B., Fernández-Bolaños, J., Heredia, A., Guillén, R., 2000. Steam-explosion pretreatment of olive cake. J. Am. Oil Chem. Soc. 77, 15–22.

Fleming, H., Walter, W., Etchells, J., 1973. Antimicrobial properties of oleuropein and products of its hydrolysis from green olives. App. Microbiology 26, 777–782.

Fernández, F.J., Villaseñor, J., Infantes, D., 2011. Kinetic and stoichiometric modelling of acidogenic fermentation of glucose and fructose. Biomass and Bioeng. 35, 3877–3883.

Fernández, M. de los Á., Espino, M., Gomez, F.J.V., Silva, M.F., 2018. Novel approaches mediated by tailor-made green solvents for the extraction of phenolic compounds from agro-food industrial by-products. Food Chem. 239, 671–678.

Ferrer, P., García-Rebollar, P., Cerisuelo, A., Ibáñez, M.A., Rodríguez, C.A., Calvet, S., De Blas, C., 2018. Nutritional value of crude and partially defatted olive cake in finishing pigs and effects on nitrogen balance and gaseous emissions. Animal Feed. Sci. Technol. 236, 131–140.

Food and Drug Administration, 2013. Standards for the growing, harvesting, packing of produce for human consumption. Available from <https://www.federalregister.gov/documents/2013/01/16/2013-00123/standards-

for-the-growing-harvesting-packing-and-holding-of-produce-for-human-consumption#p-1369> (accessed 3.29.18).

Fountoulakis, M.., Dokianakis, S.., Kornaros, M.., Aggelis, G.., Lyberatos, G., 2002. Removal of phenolics in olive mill wastewaters using the white-rot fungus *Pleurotus ostreatus*. Water Res. 36, 4735–4744.

Fu, J., Li, X., Gao, W., Wang, H., Cavaco-Paulo, A., Silva, C., 2012. Bio-processing of bamboo fibres for textile applications: a mini review. Biocatal. Biotransformation 30, 141–153.

Galli, E., Pasetti, L., Fiorelli, F., Tomati, U., 1997. Olive-mill wastewater composting: microbiological aspects. Waste Management Res. 15, 323–330.

Giannoutsou, E.P., Meintanis, C., Karagouni, A.D., 2004. Identification of yeast strains isolated from a two-phase decanter system olive oil waste and investigation of their ability for its fermentation. Bioresour. Technol. 93, 301–306.

Georgieva, T.I., Ahring, B.K., 2007. Potential of agroindustrial waste from olive oil industry for fuel ethanol production. Biotechnol. J. 2, 1547–1555.

Geventman, L.H., 1999. Solubility of selected gases in water. in; Lide, D.R. (Ed.), CRC handbook of chemistry and physics. eightieth ed. CRC Press, Florida, pp 8-86-8-90.

Grady, C.P.L., 1985. Biodegradation: its measurement and microbiological basis. Biotechnol. Bioeng. 27, 660–674.

Grosser, A., 2018. Determination of methane potential of mixtures composed of sewage sludge, organic fraction of municipal waste and grease trap sludge using biochemical methane potential assays. A comparison of BMP tests and semi-continuous trial results. Energy 143, 488–499.

Goldsmith, C.D., Vuong, Q.V., Stathopoulos, C.E., Roach, P.D., Scarlett, C.J., 2018. Ultrasound increases the aqueous extraction of phenolic compounds with high antioxidant activity from olive pomace. LWT – Food Sci. Technol. 89, 284–290.

Gonçalves, I., Silva, C., Cavaco-Paulo, A., 2015. Ultrasound enhanced laccase applications. Green Chem. 17, 1362–1374.

Gonzalez-Estrella, J., Asato, C.M., Jerke, A.C., Stone, J.J., Gilcrease, P.C., 2017. Effect of structural carbohydrates and lignin content on the anaerobic digestion of paper and paper board materials by anaerobic granular sludge. Biotechnol. Bioeng. 114, 951–960.

Gouvea, B.M., Torres, C., Franca, A.S., Oliveira, L.S., Oliveira, E.S., 2009. Feasibility of ethanol production from coffee husks. Biotechnol. Letters 31, 1315–1319.

Guerrero, A.B., Ballesteros, I., Ballesteros, M., 2018. The potential of agricultural banana waste for bioethanol production. Fuel 213, 176–185.

Gujer, W., Zehnder, A.J.B., 1983. Conversion processes in anaerobic digestion. Water Sci. Technol. 15, 127–167.

Gummadi, S.N., Panda, T., 2003. Purification and biochemical properties of microbial pectinases—a review. Process Biochem. 38, 987–996.

Hachicha, R., Rigane, H., Ben Khodher, M., Nasri, M., Medhioub, K., 2003. Effects of partial stone removal on the co-composting of olive-oil processing solid residues with poultry manure and the quality of compost. Environ. Tech. 24, 59–67.

Haagensen, F., Skiadas, I.V., Gavala, H.N., Ahring, B.K., 2009. Pre-treatment and ethanol fermentation potential of olive pulp at different dry matter concentrations. Biomass and Bioeng. 33, 1643–1651.

Hamdi, M., 1996. Anaerobic digestion of olive mill wastewaters. Process Biochem. 31, 105–110.

Hanaki, K., Matsuo, T., Nagase, M., 1981. Mechanism of inhibition caused by long-chain fatty acids in anaerobic digestion process. Biotechnol. Bioeng. 23, 1591–1610.

Hernández, V., Romero-García, J.M., Dávila, J.A., Castro, E., Cardona, C.A., 2014. Techno-economic and environmental assessment of an olive stone based biorefinery. Resour. Conserv. Recycl. 92, 145–150.

Hofrichter, M., 2002. Review: lignin conversion by manganese peroxidase (MnP). Enz. Micro. Technol. 30, 454–466.

Holliger, C., Fruteau de Laclos, H., Hack, G., 2017. Methane production of full-scale anaerobic digestion plants calculated from substrate's biomethane potentials compares well with the one measured on-site. Frontiers Energy Res. 5, 1–9.

Intelligent Energy Europe, 2008. Market of olive residues for energy. Available from <https://ec.europa.eu/energy/intelligent/projects/en/projects/more> (accessed 5.10.18).

International Olive Council, 2017. World olive oil figures. Available from <http://www.internationaloliveoil.org/estaticos/view/131-world-olive-oil-figures> (accessed 12.18.17).

IPCS INCHEM, 2005. Phenol, 4-(1,1,3,3-Tetramethylbutyl). INCHEM. Geneva.

Jackson, M.E. Fodge, D.W., Hsiao, H.Y. 1999. Effects of β-mannanase in corn-soybean meal diets on laying hen performance. Poultry Sci. 78, 1737–1741.

Jurado, E., Gavala, H.N., Baroi, G.N., Skiadas, I., 2010. Biological pretreatment and ethanol production from olive cake. 3rd International Conference on Engineering for Waste and Biomass Valorisation.

Kailis, S., Harris, D., 2007. The olive tree *olea europaea*. in: Producing table olives. Land Links, Collingwood, pp. 17–66.

Kavuma, C., 2013. Variation of methane and carbon dioxide yield in a biogas plant. Royal Institute of Technology. Available from < http://kth.diva-portal.org/smash/record.jsf?pid=diva2%3A604559&dswid=4111 >

Klinke, H.B., Olsson, L., Thomsen, A.B., Ahring, B.K., 2003. Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of *Saccharomyces cerevisiae*: wet oxidation and fermentation by yeast. Biotechnol. Bioeng. 81, 738–747.

Krich, K., Augenstein, D., Batmale, J., Benemann, J., Rutledge, B., Salour, D., 2005. Biomethane from dairy waste, in: A sourcebook for the production and use of renewable natural gas in california. Western United Dairymen, pp A1–A6.

Lafka, T.-I., Lazou, A.E., Sinanoglou, V.J., Lazos, E.S., 2011. Phenolic and antioxidant potential of olive oil mill wastes. Food Chem. 125, 92–98.

Lal, R., 2005. World crop residues production and implications of its use as a biofuel. Environ. Int. 31, 575–584.

Lazovic, B., Miranovic, K., Gasic, O., Popovic, M., 1997. Olive protein content and amino acid composition. 3rd International Symposium on Olive Growing. Available from <https://www.actahort.org/books/474/474_95.htm > (accessed 5.10.18)

Lee, C.J., Yangcheng, H., Cheng, J.J., Jane, J., 2016. Starch characterization and ethanol production of duckweed and corn kernel. Starch 68, 348–354.

Leite, A., 2015. Olive pomace pretreatments to enhance its valorization by solid-state fermentation. Universidade do Minho, Available from <http://repositorium.sdum.uminho.pt/handle/1822/41408 > (accessed on 5.10.18).

Le Floch, F., Tena, M.., Ríos, A., Valcárcel, M., 1998. Supercritical fluid extraction of phenol compounds from olive leaves. Talanta 46, 1123–1130.

Leouifoudi, I., Harnafi, H., Zyad, A., 2015. Olive mill waste extracts: polyphenols content, antioxidant, and antimicrobial activities. Adv. Pharm. Sci. 2015, 1–11.

Lesage-Meessen, L., Navarro, D., Maunier, S., Sigoillot, J.C., Lorquin, J., Delattre, M., Simon, J.L., Asther, M., Labat, M., 2001. Simple phenolic content in olive oil residues as a function of extraction systems. Food Chem. 75, 501–507.

Li, L., Feng, L., Zhang, R., He, Y., Wang, W., Chen, C., Liu, G., 2015. Anaerobic digestion performance of vinegar residue in continuously stirred tank reactor. Bioresour. Technol. 186, 338–342.

Li, Y., Zhang, R., Liu, G., Chen, C., He, Y., Liu, X., 2013. Comparison of methane production potential, biodegradability, and kinetics of different organic substrates. Bioresour. Technol. 149, 565–569.

Litaor, M.I., Khadya, N., 2018. The impact of olive mill wastewater spreading on soils using integrated approach of proximal soil survey, spatial, and multivariate analyses. Environ. Sci. Pollut. Res. 25, 7469–7478.

Luaces, P., Pérez, A.G., Sanz, C., 2003. Role of olive seed in the biogenesis of virgin olive oil aroma. J. Agri. Food Chem. 51, 4741–4745.

Maiorella, B., Blanch, H.W., Wilke, C.R., 1983. By-product inhibition effects on ethanolic fermentation by *Saccharomyces cerevisiae*. Biotechnol. Bioeng. 25, 103–121.

Makádi, M., Tomócsik, A., Orosz, V., 2012. Digestate: a new nutrient source – review. in: Kumar, S. (Ed.), Biogas. InTech, Rijeka, pp 295–306.

Marsilio, V., Di Giovacchino, L., Solinas, M., Lombardo, N., Briccoli, B., 1990. First observations on the disposal effects of olive mills vegetation wasters on cultivated soil. Acta Hortic. 286, 493–496.

Martínez-Patiño, J.C., Romero-García, J.M., Ruiz, E., Oliva, J.M., Álvarez, C., Romero, I., Negro, M.J., Castro, E., 2015. High solids loading pretreatment of olive tree pruning with dilute phosphoric acid for bioethanol production by *Escherichia coli*. Energy Fuels 29, 1735–1742.

Masghouni, M., Hassairi, M., 2000. Energy applications of olive-oil industry by-products: I. The exhaust foot cake. Biomass and Bioeng. 18, 257–262.

May, C.D., 1990. Industrial pectins: sources, production and applications. Carbohydr. Polymers 12, 79–99.

Melton, L.D., Smith, B.G., 2001. Determining the degree of methylation and acetylation of pectin, in: Wrolstad, R.E., Acree, T.E., Decker, E.A., Penner, M.H., Reid, D.S., Schwartz, S.J., Shoemaker, C.F., Smith, D., Sporns, P. (Eds.), Current protocols in food analytical chemistry. John Wiley & Sons, Inc., Hoboken, E3.4.1–E3.4.2.

Mekki, A., Dhouib, A., Sayadi, S., 2006. Changes in microbial and soil properties following amendment with treated and untreated olive mill wastewater. Microbiological Res. 161, 93–101.

Michailides, M., Christou, G., Akratos, C.S., Tekerlekopoulou, A.G., Vayenas, D.V., 2011. Composting of olive leaves and pomace from a three-phase olive mill plant. Int. Biodeterior. Biodegradation 65, 560–564.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour. Technol. 96, 673–686.

Molina Alcaide, E., Nefzaoui, A., 1996. Recycling of olive oil by-products: possibilities of utilization in animal nutrition. Int. Biodeterior. Biodegredation 38, 227–235.

Moral, P.S., Méndez, M.V., 2006. Production of pomace olive oil. Grasas y Aceites. 57, 47 – 55.

Muhammad, Arshad, Muhammad, A.Z., Muhammad, Asghar, HaqNawaz, B., 2011. Improving bio-ethanol yield: using virginiamycin and sodium flouride at a Pakistani distillery. African J. of Biotechnol. 10, 11071–11074.

Mullen, C.A., Strahan, G.D., Boateng, A.A., 2009. Characterization of various fast-pyrolysis bio-oils by NMR spectroscopy. Energy and Fuels 23, 2707–2718.

Mulat, D.G., Huerta, S.G., Kalyani, D., Horn, S.J., 2018. Enhancing methane production from lignocellulosic biomass by combined steam-explosion pretreatment and bioaugmentation with cellulolytic bacterium *Caldicellulosiruptor bescii*. Biotechnol. for Biofuels 11, 1–15.

Narendranath, N.V., Thomas, K.C., Ingledew, W.M., 2001. Effects of acetic acid and lactic acid on the growth of *Saccharomyces cerevisiae* in a minimal medium. J. Industrial Microbiology Biotechnol. 26, 171–177.

National Research Council, 2000. Biobased industrial products, priorities for research and commercialization. National Academy Press, Washington D.C.

Nayono, S.E., 2009. Anaerobic digestion of organic solid waste for energy production. Universität Karlsruhe. Available from <https://d-nb.info/999563602/34> (accessed 5.10.18).

Niaounakis, M., Halvadakis, C.P., 2006a. Characterization of olive processing waste. in: Olive processing waste management: Literature review and patent survey. Elsevier, Oxford, pp 23–64.

Niaounakis, M., Halvadakis, C.P., 2006b. Environmental effects. in: Olive processing waste management: Literature review and patent survey. Elsevier, Oxford, pp 65–80.

Niaounakis, M., Halvadakis, C.P., 2006c. Uses. in: Olive processing waste management: Literature review and patent survey. Elsevier, Oxford, pp 235–289.

Nott, K., Paquot, M., Dufour, S., Eeman, M., Deleu, M., 2009. Surface properties of new virginiamycin M1 derivatives. Colloids and Surfaces B: Biointerfaces 69, 268–275.

Numan, M.T., Bhosle, N.B., 2006. α-L-Arabinofuranosidases: the potential applications in biotechnology. J. Industrial Microbiology Biotechnol. 33, 247–260.

Obied, H.K., Allen, M.S., Bedgood, D.R., Prenzler, P.D., Robards, K., 2005. Investigation of australian olive mill waste for recovery of biophenols. J. Agri. Food Chem. 53, 9911–9920.

Obied, H.K., Bedgood, D.R., Prenzler, P.D., Robards, K., 2007. Bioscreening of australian olive mill waste extracts: biophenol content, antioxidant, antimicrobial and molluscicidal activities. Food Chem. Toxicol. 45, 1238–1248.

Olofsson, K., Bertilsson, M., Lidén, G., 2008. A short review on SSF – an interesting process option for ethanol production from lignocellulosic feedstocks. Biotechnol. Biofuels 7, 1–7.

Papaioannou, D., 1988. A method of processing waste gases from the drying of olive press-cake. Biological Wastes 24, 137–145.

Parawira, W., Murto, M., Zvauya, R., Mattiasson, B., 2004. Anaerobic batch digestion of solid potato waste alone and in combination with sugar beet leaves. Renew. Energy 29, 1811–1823.

Parida, A.K., Das, A.B., 2005. Salt tolerance and salinity effects on plants: a review. Ecotoxicol. Environ. Safety 60, 324–349.

Parr Instrument Company, 2013. 203M 1241 oxygen bomb calorimeter operating instructions. Available from < https://www.parrinst.com/products/oxygen-bomb-calorimeters/previous-calorimeter-models/> (accessed 3.29.18).

Paredes, M.J., Moreno, E., Ramos-Cormenzana, A., Martinez, J., 1987. Characteristics of soil after pollution with waste waters from olive oil extraction plants. Chemosphere 16, 1557–1564.

Patumi, M., Terenziani, S., Ridolfi, M., Fontanazza, G., 2003. Effect of fruit stoning on olive oil quality. J. American Oil Chem. Soc. 80, 249 – 255.

Paredes, C., Cegarra, J., Roig, A., Sánchez-Monedero, M.A., Bernal, M.P., 1999. Characterization of olive mill wastewater (alpechin) and its sludge for agricultural purposes. Bioresour. Technol. 67, 111–115.

Pavely, F., 2016. Senate bill – 32 California global warming solutions act of 2006: emissions limit. Available from <https://leginfo.legislature.ca.gov/faces/billTextClient.xhtml?bill_id=201520160SB32> (accessed 3.29.18).

Pelaez-Samaniego, M.R., Hummel, R.L., Liao, W., Ma, J., Jensen, J., Kruger, C., Frear, C., 2017. Approaches for adding value to anaerobically digested dairy fiber. Renew. Sustain. Energy Rev. 72, 254–268.

Pellera, F.-M., Gidarakos, E., 2016. Effect of substrate to inoculum ratio and inoculum type on the biochemical methane potential of solid agroindustrial waste. J. Environ. Chem. Eng. 4, 3217–3229.

Pellera, F.-M., Santori, S., Pomi, R., Polettini, A., Gidarakos, E., 2016. Effect of alkaline pretreatment on anaerobic digestion of olive mill solid waste. Waste Management 58, 160–168.

Peralta, R.M., da Silva, B.P., Gomes Côrrea, R.C., Kato, C.G., Vicente Seixas, F.A., Bracht, A., 2017. Enzymes from basidiomycetes—peculiar and efficient tools for biotechnology. in: Biotechnology of Microbial Enzymes. Elsevier, Oxford, pp. 119–149.

Psaltopoulou, T., Kosti, R.I., Haidopoulos, D., Dimopoulos, M., Panagiotakos, D.B., 2011. Olive oil intake is inversely related to cancer prevalence: a systematic review and a meta-analysis of 13800 patients and 23340 controls in 19 observational studies. Lipids in Health and Disease 10, 127–137.

Qureshi, N., 2009. Solvent Production, in: Schaechter, M. (Ed.), Encyclopedia of microbiology, third ed. Academic Press, Oxford, pp. 512–528.

Rabemanolontsoa, H., Saka, S., 2016. Various pretreatments of lignocellulosics. Bioresour. Technol. 199, 83–91.

Rana, G., Rinaldi, M., Introna, M., 2003. Volatilisation of substances after spreading olive oil waste water on the soil in a Mediterranean environment. Agri. Ecosystems Environ. 96, 49–58.

Ranalli, A., Serraiocco, A., 1996. Quantitative and qualitative effects of a pectolytic enzyme in olive oil production. Grasas y Aceites 4, 227 – 236.

Restuccia, D., Clodoveo, M.L., Corbo, F., Loizzo, M.R., 2018. De-stoning technology for improving olive oil nutritional and sensory features: the right idea at the wrong time. Food Res. Int. 106, 636–646.

Rodis, P.S., Karathanos, V.T., Mantzavinou, A., 2002. Partitioning of olive oil antioxidants between oil and water phases. J. Agri. Food Chem. 50, 596–601.

Roig, A., Cayuela, M.L., Sánchez-Monedero, M.A., 2006. An overview on olive mill wastes and their valorisation methods. Waste Management 26, 960–969.

Romero-García, J.M., Niño, L., Martínez-Patiño, C., Álvarez, C., Castro, E., Negro, M.J., 2014. Biorefinery based on olive biomass: state of the art and future trends. Bioresour. Technol. 159, 421–432.

Rozzi, A., Malpei, F., 1996. Treatment and disposal of olive mill effluents. Int. Biodeterior. Biodegradation 38, 135–144.

Ruggeri, B., Battista, F., Bernardi, M., Fino, D., Mancini, G., 2015. The selection of pretreatment options for anaerobic digestion (AD): a case study in olive oil waste production. Chem. Eng. J. 259, 630–639.

Sadeghi, H., Yansari, A., Ansari-Pirsari, Z., 2009. Effects of different olive cake by products on dry matter intake, nutrient digestibility and performance of Zel sheep. Int. J. Agri. 11, 39–43.

Saha, B.C., Nichols, N.N., Qureshi, N., Cotta, M.A., 2011. Comparison of separate hydrolysis and fermentation and simultaneous saccharification and fermentation processes for ethanol production from wheat straw by recombinant *Escherichia coli* strain FBR5. App. Microbiology Biotechnol. 92, 865–874.

Saha, B.C., Kennedy, G.J., Qureshi, N., Cotta, M.A., 2017. Biological pretreatment of corn stover with *Phlebia brevispora* NRRL-13108 for enhanced enzymatic hydrolysis and efficient ethanol production. Biotechnol. Progress 33, 365–374.

Sandhu, S.K., Mathur, A., Gupta, R., Puri, S.K., Adsul, M. 2018 Cellulosic Biomass-Hydrolyzing Enzymes, in: Singhania, R., Agarwal, R., Kumar, R.P., Sukumaran, R.K. (Eds.), Waste to Wealth. Springer, Singapore, pp 441–454.

Sampedro, I., Marinari, S., D'Annibale, A., Grego, S., Ocampo, J.A., García-Romera, I., 2007. Organic matter evolution and partial detoxification in two-phase olive mill waste colonized by white-rot fungi. Int. Biodeterior. Biodegradation 60, 116–125.

Sánchez, C., 2009. Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnol. Adv. 27, 185–194.

Sánchez Gómez, A.H., García García, P., Rejano Navarro, L., 2006. Elaboration of table olives. Grasas y Aceites 57. 86 – 94.

Sansoucy, R., 1985. Olive cake, in; Olive by-products for animal feed. Food & Agriculture Org. Rome. Available from < http://www.fao.org/docrep/003/x6545e/x6545e00.htm> (accessed 5.10.18).

Salas-Salvadó, J., Garcia-Arellano, A., Estruch, R., Marquez-Sandoval, F., Corella, D., Fiol, M., Gómez-Gracia, E., Viñoles, E., Arós, F., Herrera, C., 2008. Components of the mediterranean-type food pattern and serum inflammatory markers among patients at high risk for cardiovascular disease. Eur. J. Clin. Nutr. 62, 651–659.

Scollan, N.D., Choi, N.J., Kurt, E., Fisher, A.V., Enser, M., Wood, J.D., 2001. Manipulating the fatty acid composition of muscle and adipose tissue in beef cattle. British J. of Nutr. 85, 115 – 124.

Şensöz, S., Demiral, İ., Ferdi Gerçel, H., 2006. Olive bagasse (*Olea europea* L.) pyrolysis. Bioresour. Technol. 97, 429–436.

Serrano, A., Fermoso, F.G., Alonso-Fariñas, B., Rodríguez-Gutierrez, G., Fernandez-Bolaños, J., Borja, R., 2017. Olive mill solid waste biorefinery: high-temperature thermal pre-treatment for phenol recovery and biomethanization. J. Clean. Production 148, 314–323.

Servili, M., Taticchi, A., Esposto, S., Urbani, S., Selvaggini, R., Montedoro, G., 2007. Effect of olive stoning on the volatile and phenolic composition of virgin olive oil. J. Agri. Food Chem. 55, 7028–7035.

Shallom, D., Shoham, Y., 2003. Microbial hemicellulases. Current Opinion Microbiology 6, 219–228.

Silva, T.A.L., Zamora, H.D.Z., Varão, L.H.R., Prado, N.S., Baffi, M.A., Pasquini, D., 2017. Effect of steam explosion pretreatment catalysed by organic acid and alkali on chemical and structural properties and enzymatic hydrolysis of sugarcane bagasse. Waste and Biomass Valor. 1, 1–11.

Shraddha, Shekher, R., Sehgal, S., Kamthania, M., Kumar, A., 2011. Laccase: microbial sources, production, purification, and potential biotechnological applications. Enz. Res. 2011, 1–11.

Strik, D.P.B.T.B., Domnanovich, A.M., Holubar, P., 2006. A pH-based control of ammonia in biogas during anaerobic digestion of artificial pig manure and maize silage. Process Biochem. 41, 1235–1238.

Suárez, M., Romero, M.-P., Ramo, T., Macià, A., Motilva, M. J., 2009. Methods for preparing phenolic extracts from olive cake for potential application as food antioxidants. J. Agri. Food Chem. 57, 1463–1472.

Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour. Technol. 11.

Tekin, A.R., Dalgıç, A.C., 2000. Biogas production from olive pomace. Resour. Conserv. Recycl. 30, 301–313.

Thakur, B.R., Singh, R.K., Handa, A.K., Rao, M., 1997. Chemistry and uses of pectin—a review. Critical Rev. Food Sci. Nutr. 37, 47–73.

Tomati, U., Galli, E., Pasetti, L., Volterra, E., 1995. Bioremediation of olive-mill wastewaters by composting. Waste Management Res. 13, 509–518.

Topal, H., 2003. Olive cake combustion in a circulating fluidized bed. Fuel 82, 1049–1056.

Triolo, J.M., Sommer, S.G., Møller, H.B., Weisbjerg, M.R., Jiang, X.Y., 2011. A new algorithm to characterize biodegradability of biomass during anaerobic digestion: influence of lignin concentration on methane production potential. Bioresour. Technol. 102, 9395–9402.

Tufarelli, V., Introna, M., Cazzato, E., Mazzei, D., Laudadio, V., 2013. Suitability of partly destoned exhausted olive cake as by-product feed ingredient for lamb production. J. Animal Sci. 91, 872–877.

United States International Trade Commission, 2013. Olive oil: conditions of competition between U.S. and major foreign supplier industries. Available from <https://www.usitc.gov/publications/332/pub4419.pdf> (accessed 5.10.18)

van Lier, J.B., Mahmoud, N., Zeeman, G., 2008. Anaerobic wastewater treatment. in: Henze, M., van Loosdrecht, M.C.M., Ekama, G.A., Brdjanovic, D. (Eds.), Biological wastewater treatment: Principles, modelling and design. IWA Publishing, London, pp 402–440.

van Zyl, W.H., Rose, S.H., Trollope, K., Görgens, J.F., 2010. Fungal β-mannanases: mannan hydrolysis, heterologous production and biotechnological applications. Process Biochem. 45, 1203–1213.

Vierhuis, E., Schols, H.., Beldman, G., Voragen, A.G.., 2000. Isolation and characterisation of cell wall material from olive fruit (Olea europaea cv koroneiki) at different ripening stages. Carbohydr. Polymers 43, 11–21.

Vierhuis, E., Servili, M., Baldioli, M., Schols, H., Voragen, A., 2001. Effect of enzyme treatment during mechanical extraction of olive oil on phenolic compounds and polysaccharides. J. Agri. Food Chem. 49, 1218 – 1223.

Vlyssides, A.G., Bouranis, D.L., Loizidou, M., Karvouni, G., 1996. Study of a demonstration plant for the co-composting of olive-oil-processing wastewater and solid residue. Bioresour. Technol. 56, 187–193.

Vlyssides, A., Loizidou, M., Gimouhopoulos, K., Zorpas, A., 1998. Olive oil processing wastes production and their characteristics in relation to olive oil extraction methods. Fresenius Environ. Bull. 7, 308–313.

Vossen, P., 2007. Olive oil: history, production, and characteristics of the world's classic oils. Hortsci. 42, 1093–1100.

Vyas, S., Chhabra, M., 2017. Isolation, identification and characterization of *Cystobasidium oligophagum* JRC1: a cellulase and lipase producing oleaginous yeast. Bioresour. Technol. 223, 250–258.

Walker, M., Zhang, Y., Heaven, S., Banks, C., 2009. Potential errors in the quantitative evaluation of biogas production in anaerobic digestion processes. Bioresour. Technol. 100, 6339–6346.

Wilkie, A.C., 2008. Biomethane from biomass, biowaste, and biofuels. in: Wall. J., Harwood, C.S., Demain, A, (Eds.), Bioenergy, American Society of Microbiology, Washington D.C., pp. 195–205.

Woo, J.-M., Yang, K.-M., Kim, S.-U., Blank, L.M., Park, J.B., 2014. High temperature stimulates acetic acid accumulation and enhances the growth inhibition and ethanol production by *Saccharomyces cerevisiae* under fermenting conditions. App. Microbiology Biotechnol. 98, 6085–6094.

Wood, S., Cowie, A., 2004. A review of greenhouse gas emissions factors for fertilizer production. IEA Bioenergy Task 38. Available from < https://www.ctc-n.org/resources/review-greenhouse-gas-emission-factors-fertiliser-production> (accessed 5.10.18)

Ximenes, E., Kim, Y., Mosier, N., Dien, B., Ladisch, M., 2010. Inhibition of cellulases by phenols. Enz. Micro. Technol. 46, 170–176.

Yablochkova, E.N., Bolotnikova, O.I., Mikhailova, N.P., Nemova, N.N., Ginak, A.I., 2003. specific features of fermentation of D-xylose and D-glucose by xylose-assimilating yeasts. App. Biochem. Microbiology 39, 302–306.

Yadav, S., Yadav, P.K., Yadav, D., Yadav, K.D.S., 2009. Pectin lyase: a review. Process Biochem. 44, 1–10.

Ye, R., Jin, Q., Bohannan, B., Keller, J.K., Bridgham, S.D., 2014. Homoacetogenesis: a potentially underappreciated carbon pathway in peatlands. Soil Biology Biochem. 68, 385–391.

Zanichelli, D., Carloni, F., Hasanaj, E., D'Andrea, N., Filippini, A., Setti, L., 2007. Production of ethanol by an integrated valorization of olive oil byproducts. Environ. Sci. Pollut. Res. 14, 5–6.

Zheng, Y., Pan, Z., Zhang, R., 2009. Overview of biomass pretreatment for cellulosic ethanol production. Int. J. Agri. Biological Eng. 2, 51–68.