Biorefining for olive wastes management and efficient bioenergy production

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Abstract

The potential of olive wastes for development of a multi-product biorefinery was investigated. Different parts of olive wastes, i.e., stone, pomace, leaves, and wood, were subjected to liquid hot water, organosolv, and acid-catalyzed organosolv (ACO) pretreatments prior to bioconversion through three different scenarios. The first scenario, i.e., anaerobic digestion of substrates for biogas production, yielded 219.3 m^3 biomethane per hectare of olive trees, equated to 247.4 L gasoline. The highest methane production of 103.3 m^3 was attributed to liquid hot water pretreated wood and ACO increased methane yield for leaf and stone samples by 200 and 33%, respectively. The second scenario, i.e., fermentation of wastes for bioethanol production, resulted in 295.9 L bioethanol per hectare of olive trees, equivalent to 196.1 L gasoline. Organosolv pretreated wood with 82.9% production yield and 152.5 L bioethanol constitutes this plan's dominant part. The ACO pretreatment improved fermentation yield for pomace and stone samples by 49% and 53%, respectively. The third scenario, included the utilization of olive wastes in bioethanol production, anaerobic digestion of fermentation residues, and lignin separation, resulted in 295.9 L bioethanol, 137.2 m^3 biomethane, and 347.1 kg lignin, equated with 521.6 L gasoline. Furthermore, the remaining oil content in pomace and stone samples was 17% and 20%, respectively, which could be used for biodiesel production. Overall, olive wastes processing through an integrated biorefinery plant with multiple products significantly improved the energy recovery of the whole plant.

***Keywords*:** Biorefinery; Olive wastes; Bioethanol; Biogas; Organosolv pretreatment.

1. Introduction

Olive and its byproducts are a significant part of the daily diet worldwide, and its nutritional value, along with health-promoting properties, has been investigated in many studies recently [1]. Consequently, olive trees have been widespread all over the world, so that more than 10 million hectares were cultivated in 2018 and produced over 21 million tonnes of olive and 3 million tonnes of olive oil [2,3]. Olive trees need to be pruned annually by removing unproductive branches to enhance production in the next harvest [4], and the amount of olive tree pruning biomass (OTPB) was estimated at around 3 ton/ha [5]. Therefore, a broad spectrum of lignocellulosic biomass, including OTPB, stones from table olive production, and olive pomace from oil extraction units, are available in an olive tree field and its processing [6]. While these materials are an abundant source of renewable, low-cost biomass for the production of biofuels, energy, and a variety of valuable chemicals [7], they are managed by burning or scattering on fields as fertilizer, which both methods cause serious environmental issues, e.g., CO_2 emission and propagation of vegetal diseases [8].

Besides, industrial developments worldwide, accompanied by concerns over fossil fuel consumption and environmental issues, have intensified the need for alternative, sustainable, and inexpensive energy resources [9]. Thus, efficient exploitation of lignocellulosic biomass as biofuels, e.g., bioethanol and biomethane, could meet the demand for energy sources and provide strategies for environmental conservations [10]. However, the intrinsic recalcitrance of these resources hinders the development of sustainable technology for utilizing them [11]; thus, a pretreatment stage seems indispensable to modify the structure of biomass and improve biodegradability [12]. However, it is the most costly stage that influences the cost of both previous and subsequent operations [13]. In fact, the primary impediment toward widespread

bioenergy production is the deficiency of cost-effective technology for overcoming biomass recalcitrance [14] because biofuels have to be cost-competitive with fossil fuels in the market. Therefore, the application of an efficient pretreatment method throughout an integrated biorefinery plant is suggested to address the economy of the process [15].

In a biorefinery plant, biomass is converted into multiple chemicals and energy products through an integrated method; thereby, it can increase the commercial value of the substrate while managing the waste streams [16]. The primary purpose of the biorefinery is to improve the economy by producing the maximum potential valuable byproducts and reduce the wastes [17]. In this regard, organosolv pretreatment is one of the most promising methods for developing a lignocellulose biorefinery, as it is able to fractionate lignocellulosic wastes into three separate processes streams, including cellulose-rich solid, a relatively pure lignin precipitate, an aqueous mixture of hemicelluloses and extractives [18]. Specifically, using ethanol in organosolv processes has additional benefits such as low toxicity to both environment and subsequent bioconversions and the ease of solvent recovery that reduce operating costs [19].

Bioethanol is a renewable fuel with remarkable characteristics, including low toxicity to the environment, high octane number, and significant energy content, making it a suitable alternative fuel for long and near-term applications [20]. Furthermore, a diverse range of renewable chemicals, such as plastics and polyethylene, can be obtained from bioethanol [21].

After hydrolysis and fermentation, the produced ethanol is recovered, and solids (containing lignin, unreacted cellulose and hemicellulose, enzyme, and organisms' debris) remain. The remaining materials are subjected to different processes, including landfill, burning, and anaerobic digestion [22].

Anaerobic digestion (AD) is one of the economic and lowest energy-consuming processes that produce considerable amounts of bioenergy along with waste management. Organic wastes in landfills decompose and release methane and carbon dioxide into the atmosphere. This natural process causes environmental pollution because the serious global warming potential of methane is evaluated to be 20 times greater than carbon dioxide. Burning the wastes is also accompanied by different environmental impacts. The feedstock composition has a significant effect on biogas content and methane yield. Fats improve the methane yield, while carbohydrates and proteins have much faster conversion rates but lower methane yield [23]. Different parts of olive wastes contain considerable amounts of fat, protein, and carbohydrates [24], making the waste suitable for biogas production.

Lignin is a phenolic polymer placed in the plant cell wall, causing rigidity and strength. Moreover, producing high-value compounds from lignin, like antioxidants, surfactants, and emulsifiers, has been investigated recently [25]. Valorization of lignin makes a considerable improvement in the viability and sustainability of a biorefinery. Besides, separation of less altered lignin is an exclusive feature of organosolv pretreatment. Therefore, organosolv lignin with low molecular weight and the least contaminant content (carbohydrates, ash) and high heating value is an appropriate solid fuel for burning [26,27].

Olive tree wastes have received significant attention recently. Most studies have accomplished obtaining antioxidant phenolic compounds from olive leaves [7] or olive mill wastewater [28-31]. Valorization of OTP mixture into single products such as ethanol, biogas, and lignin through steam explosion [32, 33], combination of acid and alkaline pretreatments [4], high-temperature thermal pretreatment [34], and acid-catalyzed LHW [35] were investigated. However, to our knowledge, separate utilization of all kinds of olive wastes produced in the field and its

processing in one integrated plant for both bioethanol and biogas production along with lignin separation as a value-added product has not been investigated yet. Assessing the effect of organosolv pretreatment on various olive waste bioconversions, comparing LHW, organosolv, ACO, and employing non-isothermal simultaneous saccharification and fermentation (NSSF) method for bioethanol production from these wastes are also missing in previous studies.

This study aimed to maximize the produced energy from olive wastes through a biorefinery approach. Liquid hot water (LHW), organosolv, and acid-catalyzed organosolv (ACO) pretreatments were conducted, lignin was separated, and pretreated solids were used for bioethanol and biogas production. The most efficient plan for bioenergy production was investigated through the evaluation of three different scenarios. The first scenario was the anaerobic digestion of all substrates. The second studied scenario was the conversion of all substrates to bioethanol, and the third scenario included the utilization of olive wastes in bioethanol production along with AD of fermentation residues. The overall gasoline equivalent of all scenarios was calculated to identify the most efficient biorefinery plant.

2. Materials and methods

2.1. Raw materials

Olive tree pruning biomass, from Zard cultivar, *Olea europaea* species trees, was collected from Isfahan University of Technology forests, Isfahan, Iran (32.720362, 51.534890). Afterward, the OTP lot was separated into distinct portions of leaf and wood wastes. The olive fruits were divided into two groups; one was crushed in a cold press apparatus to obtain edible oil, which leaves a wet pomace after oil extraction. The other was sent to the table olive production plant to remove stones. Wood, leaves, pomace, and removed stones were separated and air-dried. All

samples were milled, sieved through 20-80 mesh to achieve particle size less than 1 mm, placed in a plastic bag, and stored at room temperature until use.

2.2. Lignocelluloses pretreatments

Three different pretreatments, including liquid hot water (LHW), organosolv, and acid-catalyzed organosolv (ACO), were conducted on all prepared substrates, following a procedure described by Obama et al. [36]. In brief, all pretreatments were performed at the solid to liquid ratio of 1:8 and 180℃ for 1 hour. Organosolv pretreatment was performed with 75% aqueous ethanol, and 1.0% sulfuric acid was added to 75% aqueous ethanol in the acid-catalyzed organosolv pretreatment. The mixture was loaded in a 500 mL stainless steel reactor (Kayhan Steel Sanat Co., Isfahan, Iran) equipped with a pressure gauge and thermometer and heated up to the desired temperature at the rate of 4℃/min using an oil bath (One 10, Memmert, Germany). Afterward, the reactor cooled to room temperature in an ice bath. Treated solids were filtered, and then two sets of triple washing steps were conducted; first with 300 mL (3×100) ethanol at 60℃ and the second time by water 60℃ to remove the remaining solvent. Washes were combined with filtrates. Three volumes of water were added to the effluent mixture for the sedimentation of dissolved lignin. The lignin precipitate was collected on Whatman No.1 filter paper, washed, air-dried, and kept in zip-keep bags until analysis.

2.3. Enzyme hydrolysis

The enzymatic hydrolysis of olive wastes was conducted in 118 mL glass bottles with 50 g/l solid substrates soaked in 0.05 M sodium citrate buffer. The initial pH was adjusted to 4.8, and 0.5 g/l sodium azide was added to the mixture to prevent microbial growth [37]. Samples were autoclaved at 121℃ for 20 min. Afterward, 15 FPU of Cellic® CTec2 (Novozymes, Denmark) with 125 FPU/mL activity was loaded to bottles under the microbial hood and incubated at 45℃

for 72 h with 120 rpm. The enzyme activity was measured through the method presented by Adney and Baker [38]. Samples were taken periodically and stored in the freezer until sugar analysis. The hydrolysis yield was calculated according to Eq.1 [39].

$$\text{Hydrolysis yield (\%)} = \frac{Produced\ glocuse\ (\frac{g}{L})}{Glucan(\%) \times Cs \times 1.11} \times 100 \qquad \text{(Eq.1)}$$

Where C_s refers to the substrate concentration in 1 liter of buffer solution and 1.11 is the hydration factor for conversion of glucan to glucose [39].

2.4. Non-isothermal simultaneous saccharification and fermentation (NSSF)

All treated and untreated substrates were subjected to ethanolic fermentation using the NSSF method [40]. A solution of 50 g/L solid substrates with all necessary nutrients for cell growth in 0.05 M sodium citrate buffer at pH 4.8 was prepared in 118 mL glass bottles [41]. The samples were autoclaved at 121℃ for 20 min, and 15 FPU enzyme was added when the temperature settled down. Then, the samples were incubated at 45℃ for 24 h at 120 rpm. Afterward, the temperature was reduced to 37℃, 10 g/l yeast was loaded into each bottle. The yeast, *Saccharomyces cerevisiae* (CCUG 53310, Culture Collection, University of Gothenburg, Sweden), was prepared according to the Karimi et al. [42] method. The samples were sealed and flushed with pure nitrogen to provide anaerobic conditions and incubated for 72 h in a shaker incubator at 120 rpm. Liquid samples were taken periodically and kept in zip-keep bags in the freezer until ethanol concentration analysis. After completion of the test, all reactors' content was freeze-dried (Christ, Alpha 1-2 LDplus Model, Germany) separately, and dried samples were collected to use as a feedstock for biomethane production.

The bioethanol production yield was calculated using Eq.2 [43].

$$\text{Ethanol yield (\%)} = \frac{Produced\ ethanol\ (\frac{g}{L})}{Glucan(\%) \times Cs \times 1.11 \times Ye} \times 100 \qquad \text{(Eq. 2)}$$

where Y_e indicates the theoretical yield of ethanol production from glucose, i.e., 0.51.

2.5. Anaerobic digestion

Biological methane production was carried out at mesophilic conditions in 118 mL dark serum glass bottles through the method presented by Hansen et al. [44]. All pretreated and untreated samples and fermentation residues were anaerobically digested using an inoculum obtained from the 7000 m^3 anaerobic digester operating at 37°C in Isfahan Municipal Wastewater Treatment plant, Isfahan, Iran. Solid substrate (0.25 g), 20 mL inoculum, and 5 mL distilled water were loaded to each reactor to attain the inoculum to substrate ratio of 2 (based on volatile solid content) [45]. The bottles were sealed with rubber stoppers and aluminum caps, and the headspace of each sample was flushed with pure N_2 for about 2 min. A blank sample containing the same amount of inoculum and distilled water (without substrate) was also prepared to determine inoculum's methane production. After that, all samples were incubated at 37 °C for 45 days and manually mixed once a day. All experiments were conducted in duplicate. Gas samples were taken once every three days at first 15 days and then every five days until the remaining days, using a 200 µL gas pressure lock syringe (VICI, Precision Sampling Inc., USA) and the methane content of samples analyzed by gas chromatography. The volume of produced biogas was evaluated through the water displacement method [46].

2.6. Analytical methods

The compositional changes in substrates caused by pretreatments were analyzed through the two-step acid hydrolysis method described by National Renewable Energy Laboratory (NREL) [47]. Furthermore, the extractive content of olive wastes and the dry weight of all prepared substrates were also evaluated [48,49]. The pomace and stone samples' oil content was determined through the Perdomo et al. [50] method by a Soxhlet extractor using n-hexane solvent.

Changes in the crystallinity index and chemical structure were estimated using Fourier transform infrared (FTIR) spectrometer (WQF-510A FTIR, BRAIC, China). Collected spectra included an average of 32 scans with a resolution of 4 cm^{-1} in the range of 400 cm^{-1} to 4400 cm^{-1}.

The morphological changes in the substrate surface were studied by scanning electron microscopy (SEM). All samples were coated with gold, and the images were captured by SEM (EvoLs, Zeiss, Germany) at 15 kV.

The concentration of sugars and ethanol was measured by a high-performance chromatography (HPLC) equipped with UV-Vis and RI detectors. An Aminex HPX-87H column (Bio-Rad, Richmond, CA, USA) with 0.6 mL/min eluent of 5 mN sulfuric acid at 60 ℃ was used for ethanol concentration determination. For evaluating the concentration of glucose, xylose, arabinose, and mannose, Aminex HPX-87P column (Bio-Rad, Richmond, CA, USA) with 0.6 mL/min deionized water as a mobile phase at 85 ℃ was used.

A gas chromatograph (GC) (SP-3420A, Beijing Beifen Ruili Analytical Instrument Co., Beijing, China) equipped with a thermal conductivity detector (TCD) operating at 150 ℃ was employed for determination amount of carbon dioxide and methane produced in anaerobic digestion. The GC was equipped with a packed column (3 m length and 3 mm internal diameter, stainless steel, Porapak Q column, Chrom- pack, Germany), and nitrogen with a 20 mL/min flow rate was used as a carrier gas. The injection temperature was set at 100 ℃.

2.7. Statistical analysis

Analysis of variance (ANOVA) was conducted on experimental results to evaluate significant differences between the results using SAS 9.4 software using the Tukey method with a 95% confidence limit. There are no significant differences between groups with the same letters at a 5% probability (P-value < 0.05).

2.8. Gasoline equivalent

To compare different scenarios, the gasoline equivalents of all main products and byproducts were calculated [51]. One hectare of olive trees was considered as a basis of all calculations, and lower heating values of fuels (at 25°C) were considered 36.1 MJ/Nm^3 for biomethane, 21.2 MJ/L for ethanol, and 32.0 MJ/L for gasoline. Higher heating values (HHV) of lignin samples were also measured using a bomb calorimeter according to ASTM D2015 standard method [52]. These values were obtained as 16.8±0.3, 21.2±0.6, 14.1±0.8, and 9.1±0.7 MJ/kg for leaves, stone, pomace, and wood samples, respectively. TS, VS, and solid recoveries of substrates after pretreatments were also applied in calculations.

3. Results and discussion

Olive fruit and plant wastes were subjected to LHW, organosolv, and ACO pretreatments, and the pretreated solids were subjected to AD and ethanol production. Three scenarios were defined to determine the most efficient plan for designing an olive wastes-based biorefinery. The first scenario included the biogas production from all substrates. The second was the ethanolic fermentation of all substrates, and the third studied scenario was the bioethanol production from olive wastes and anaerobic digestion of fermentation residues. The overall gasoline equivalent values for each scenario were evaluated to compare the bioenergy recovery from the wastes.

3.1. Effect of pretreatments on substrates' compositions and structures

The chemical composition of all untreated and pretreated substrates, water and ethanol extractive content, and solid recoveries are presented in Table 1. As shown in Table 1, untreated olive wastes contained 32-54% glucan, 30-46% hemicellulosic sugars (8-23% xylan), and 18-30% lignin. Since the hemicellulose and lignin together constitute a relatively high fraction of

248 untreated substrates, a pretreatment step is necessary to make the glucan the dominant part of
249 these wastes and enzymatically digestible.
250 Rodríguez et al. [24] reported untreated olive stone composition to be 36.4% glucan, 26.8%
251 xylan, and 26.0% lignin, which is close to the results of the present study. On the other hand,
252 Lama et al. [53], evaluating the extraction of antioxidants and fermentable sugars from olive
253 stone, indicated that olive stone comprises 19.2% glucan, 28.6% xylan, and 37.2% lignin. Since
254 the composition of olive wastes is considerably affected by many factors like cultivar, growth
255 condition, irrigation, and climate [54], the resemblance and difference of wastes' composition
256 throughout different regions could be explained.

257 All conducted pretreatments affected the composition of olive wastes to some extent.
258 Considering Table.1, ACO and organosolv pretreatments resulted in pretreated solids with 25-
259 52% and 17-34% lower lignin content, respectively. The highest delignification yield of 52%,
260 attributed to ACO pretreated stone samples, led to the release of 151.9 mg lignin per g substrate
261 into the liquor. Furthermore, organosolv treatment reduced the biomass recalcitrance to such an
262 extent that it almost tripled the lignin removal of leaf and pomace samples compared to LHW.
263 Dissolution of lignin into organic liquor is the major advantage of ACO at first and organosolv
264 pretreatment afterward because acid catalyst addition accelerates breaking of glycosidic bonds
265 for hemicellulose and lignin-hemicellulose linkages, so the delignification process improves due
266 to increasing pretreatment severity [55,56].

267 Similarly, Kumari and Singh [57] revealed that due to the capability of organosolv pretreatment
268 for disrupting the internal bonds of lignin and hemicellulose, it would be an efficient method for
269 the treatment of lignocellulosic biomass.

270

Table 1

Chemical composition, extractive content, and solid recovery of untreated and pretreated wastes.

Pretreatment conditions		Water extractives (%)	Ethanol extractives (%)	Solid recovery (%)	Glucan (%)	Xylan (%)	Other sugars (%)	Lignin (%)
T (°C)	Time (min)							
180	60							
Olive leaves								
Untreated		39.4±1.5	6.7±1.2	-	54.7±1.4	8.6±0.7	21.3±0.2	18.6±0.3
Liquid hot water				46.4±0.9	60.8±1.2	3.4±0.2	9.5±0.5	36.2±0.5
Organosolv				42.2±1.5	44.5±0.9	12.1±0.2	15.2±0.3	32.7±0.2
Acid-catalyzed organosolv				40.8±1.8	48.9±1.1	4.1±0.2	17.3±0.1	30.6±1.5
Olive wood								
Untreated		23.8±1.7	37.7±2.5	-	32.6±0.5	15.7±0.3	30.4±0.3	19.4±0.3
Liquid hot water				59.7±2.6	45.2±1.7	5.5±0.6	22.8±0.5	29.7±0.6
Organosolv				54.0±2.1	47.6±1.3	11.0±0	23.9±0.2	24.2±0.1
Acid-catalyzed organosolv				57.1±1.8	48.2±1.3	13.2±0.3	18.7±0.3	19.6±0.3
Olive stone								
Untreated		17.7±1.6	18.1±1.9	-	38.5±0.9	23.5±1.5	9.9±0.6	28.7±0.5
Liquid hot water				71.2±1.5	47.9±1.2	4.3±0.5	9.1±1.9	38.2±0.6
Organosolv				62.1±2.1	48.3±1.6	13.2±1.1	14.1±0.7	30.2±1.3
Acid-catalyzed organosolv				47.6±2.4	62.8±0.4	9.1±0.9	7.4±0.3	28.4±0.9
Olive pomace								
Untreated		12.6±2.2	22.3±1.8	-	40.4±1.1	20.4±0.5	15.3±0.3	30.2±1.5
Liquid hot water				70.2±1.5	40.3±0.8	7.7±1.5	16.7±0.4	41.2±1.2
Organosolv				67.4±1.6	38.6±0.5	4.8±0.4	18.3±1.1	37.3±1.4
Acid-catalyzed organosolv				65.9±1.4	48.3±0.2	10.1±0.7	12.2±1.4	34.3±0.2

LHW and ACO pretreatments removed 71% and 76% of the hemicellulose content of stone sample, respectively. Likewise, both methods reduced the hemicellulose fraction of wood and pomace by about 60%. Nevertheless, the maximum xylan removal of 81% was achieved by LHW for leaf samples. Previous studies confirmed that LHW acts like an acid-catalyzed pretreatment at high temperatures because the deacetylation of hemicellulose chains results in the

279 formation of acetic acid and the concentration of acetic acid considerably increases as a
280 consequence of boosted hemicellulose breakdown at 180℃ [35]. Hashemi et al. [58] illustrated
281 that LHW pretreatment at 180 ℃ for 60 min reduced the xylan content from 19.2 to 2.6% and
282 improved the ethanol yield consequently. As a result of hemicellulose reduction lignin content
283 for all samples was increased, which was also observed by Momayez et al. [59].
284 Compared to hemicellulose, lignin and glucan were more resistant against LHW degradation, so
285 that the maximum delignification yield was 9% for the leaf sample. In the case of glucan
286 recovery, most of the cellulose remained in solid fraction for all samples. The glucan recovery
287 was higher in LHW, varied between 51-88%, and the best glucan recovery was achieved by 88%
288 for LHW pretreated stone samples. Since more glucan loss results in lower solid recovery, solid
289 recoveries in Table 1 have descending order from LHW to acid-catalyzed organosolv
290 pretreatment [60]. The average recovery for organosolv and ACO was 70%, except for the leaf
291 sample, which was accompanied by lower glucan recoveries (34% for organosolv and 36% for
292 ACO pretreatment). Sannigrahi et al. [61] investigated the effect of organosolv pretreatment on
293 enzymatic hydrolysis as well as cellulose structure and crystallinity. They ascribed the glucan
294 loss to the degradation of glucomannans and to some extent, cellulose. In fact, the glucan share
295 that existed in raw material was divided into three sectors: near 79% was recovered in pretreated
296 solid, about 3% was detected as glucose in the liquid phase, and the rest is inferred to have
297 degraded to furans, acetic acid, and other degradation products, which are soluble in pretreatment
298 liquor.
299 As shown in Table 1, olive wastes contain a considerable amount of water and ethanol
300 extractives. Due to the complexity and variety of extractives composition, they may have a
301 different effect on enzymatic hydrolysis, fermentation, or anaerobic digestion [62]. Further study

302 is recommended to investigate whether olive wastes extractives improved the mentioned
303 processes or inhibit them.

304 The surface morphology changes of pretreated and raw materials were investigated using a
305 scanning electron microscope (SEM). As shown in Figure 1, untreated substrates have a non-
306 porous, smooth, and inaccessible structure which turned to beehive-like cells with rough and
307 extended internal surface after pretreatment. These changes are attributed to lignin and
308 hemicellulose removal [55]. Oliveira et al. applied a two-stage process on sugarcane straw,
309 including a hydrothermal pretreatment and an alkaline delignification step; the captured SEM
310 images indicated that although LHW fragmented the well-ordered structure of the substrate, the
311 chemical pretreatments have more severity to open up lignocellulose fibers [63]. The recent
312 statement is conceivable through a comparison of different parts of Figure 1.

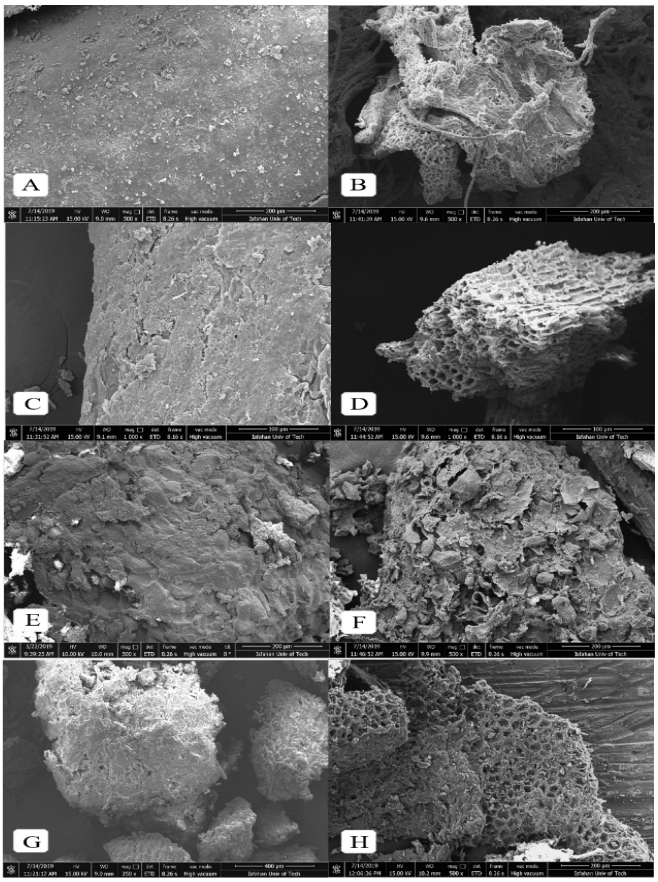


Figure 1. Scanning electron micrographs (SEM) of (A) Uuntreated leaves, (B) ACO pretreated leaves, (C) untreated woods, (D) organosolv pretreated woods, (E) untreated stones, (F) ACO pretreated stones, (G) untreated pomace, and (H) liquid hot water pretreated pomace.

The chemical structure variation in olive wastes was investigated by FTIR, absorptions along with the corresponding wavenumbers, and the functional groups are presented in Tables S1-S4 in supplementary data. The crystallinity index (CI) and Total crystallinity index (TCI), which are defined as a ratio of A_{1430}-A_{898} and A_{1375}-A_{2915} [55], are shown in Table 2. Elimination of amorphous regions (i.e., hemicellulose) through pretreatments increased cellulose content in most cases. CI has been augmented in some samples, indicating the removal of amorphous

cellulose. The highest TCI reduction (37%) attributed to ACO pretreated pomace, implying the conversion of high-crystalline cellulose to amorphous type [64].

Table 2

Crystallinity index and total crystallinity index of untreated and pretreated substrates

Substrates	CI	TCI
Olive leaves		
Untreated	2.05	1.11
Liquid hot water	2.07	1.03
Organosolv	1.68	1.01
Acid-catalyzed organosolv	1.77	1.03
Olive wood		
Untreated	1.98	0.93
Liquid hot water	1.84	1.07
Organosolv	1.65	1.05
Acid-catalyzed organosolv	1.65	1.09
Olive stone		
Untreated	1.43	1.00
Liquid hot water	2.04	0.82
Organosolv	1.51	1.09
Acid-catalyzed organosolv	1.46	0.98
Olive pomace		
Untreated	1.79	0.86
Liquid hot water	1.92	0.83
Organosolv	1.45	0.96
Acid-catalyzed organosolv	1.50	0.93

3.2. Enzymatic hydrolysis

Enzymatic digestibility of both untreated and pretreated olive wastes was evaluated by the hydrolysis process. Figure 2 represents the hydrolysis yields after 72 h. As expected, the

hydrolysis of untreated samples was not efficient enough, and amounts of 17.6%, 14.8%, 21.1%, and 37.8% for leaf, stone, pomace, and wood obtained, respectively. All conducted pretreatments enhanced hydrolysis yield for all substrates and resulted in hydrolysates with 4.2-38.6 g/L higher glucose concentration. As shown in Figure 2, organosolv boosted hydrolysis yield over 3 times and caused the highest glucose yield of 53.5% for olive stone, leading to the release of 322.2 mg glucose/g stone into hydrolysate. Further, with about 80% rise in the hydrolysis of pomace and leaf samples, organosolv is recognized as a promising pretreatment method. Moreover, ACO was the most effective pretreatment for wood and pomace samples with 41% and 101% yield increments, respectively. Notwithstanding the fact that ACO improved leaf hydrolysis by 72%, the ANOVA results denoted that there is no statistically significant difference between glucose yields of organosolv and ACO pretreated leaf ($p < 0.05$). In general, organosolv and ACO pretreatments improve hydrolysis yield by disrupting internal lignin bonds, lignin-hemicellulose bonds, and ether linkages [65]. Compared to other performed pretreatments on olive stone, ACO was not efficient enough, and the pseudo-lignin formation hypothesis has been responsible for this fact. Recently, many studies [35,66,67] have revealed that lignin from ACO and LHW in high temperatures and low pH conditions could redeposit on cellulose surface and hinder enzyme accessibility. In accordance with the recent hypothesis, the surface of the ACO pretreated stone samples (Figure 1-F) is covered with some spherical lignin-like materials, which may constrain enzyme accessibility. On the other hand, LHW prior to enzymatic hydrolysis resulted in 17-48% higher glucose yield. LHW can remove the acetyl groups of hemicellulose and release them to form acetic acid, which further accelerates hemicellulose decomposition and improves glucose yield [68]. This deacetylation process increases enzymatic digestibility through both formations of more easily digestible xylo-oligomers with fewer branches and alleviation of steric restrictions

provoked by acetyl groups in cellulase accessibility to cellulose [35]. To summarize, as can be seen in Figure 1, all pretreatments have increased accessible surface area through mentioned modifications and thus have improved enzymatic hydrolysis.

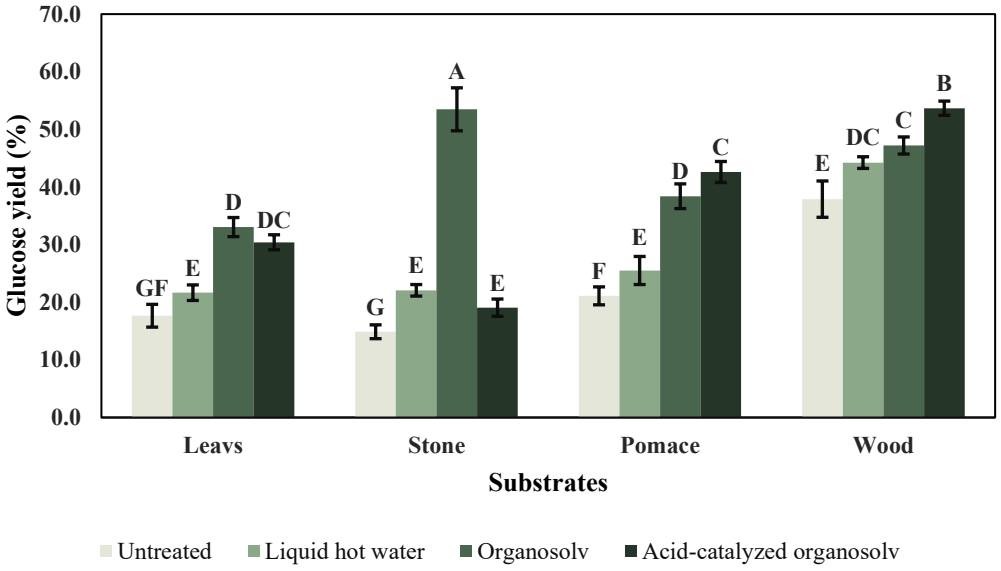


Figure 2. Glucose yields in enzymatic hydrolysis of untreated and pretreated samples, Different letters indicate a significant difference between hydrolysis yields. There is a significant difference between columns with different letters. Standard deviations are reported as error bars.

3.3. Scenario I: Biogas plant based on olive wastes

The second scenario included the conversion of untreated and pretreated olive wastes to biogas followed by lignin separation through organosolv and ACO pretreatments as a byproduct. The results of anaerobic digestion are presented in Figure 3. AD of untreated wastes accompanied by 87.4-166.8 mL/gVS produced methane. Among these, the productivity of untreated pomace and stone-with 17% w/w and 20% w/w oil content- resulted in 166.8 and 140.9 mL/gVS biomethane, which was significantly higher than the others. As mentioned before, feedstock composition

370 significantly affects methane production, so the difference between the amounts of obtained
371 methane from various untreated substrates is conceivable. Many studies indicated that digestion
372 of high-lipid content materials improves biomethane production considerably because
373 theoretically, the methane potential of lipids (1014 L/kgVS) is much higher than that of
374 carbohydrates (e.g., 370 L/kgVS for glucose) [69]. Angelidaki and Ahring [70] comparatively
375 studied the digestion of pure manure and co-digested manure with 5% olive oil wastewater and
376 fish oil; the methane yield had a two-fold increase with lipid addition. Hashemi et al. [58] have
377 also corroborated the effect of remaining oil in safflower seed cake on methane productivity at
378 the first 20 days of digestion.

379 On the other hand, untreated leaf and wood samples were less efficient, which may be related to
380 their high extractive content (Table 1). Olive wastes are a rich source of extractable phenolic
381 compounds [34]. Although these compounds' inhibitory effect depends on their concentration
382 and physicochemical characteristics, many studies claimed that they have an inhibitory effect on
383 AD [71]. Borja et al.[72] added various concentrations of the most important phenolic
384 constituents of olive mill wastewaters to anaerobic digestion bottles to investigate whether they
385 fermented to methane or inhibited the digestion process. The presented results indicate
386 oleuropein at 600 mg L^{-1}, caffeic, protocatechuic acids, and p-hydroxybenzoic at 1000 mg L^{-1},
387 and tyrosol at ≥2000 mg L^{-1} inhibited the methane production.

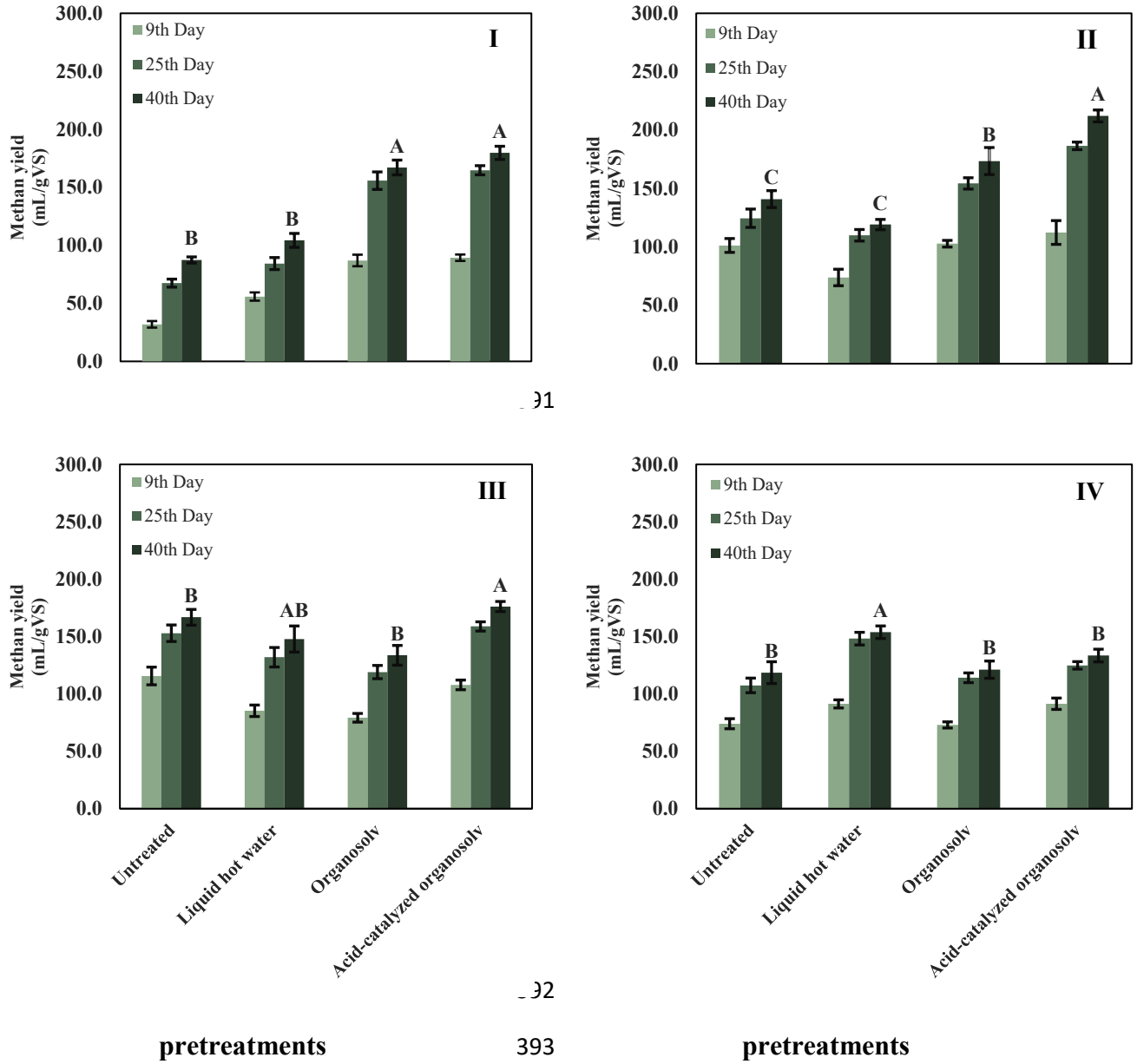


Figure 3. Methane production yields for (I) leaves, (II) stone, (III) pomace, and (IV) wood after 9 days, 25 days, and 40 days anaerobic digestion. Standard deviations are reported as error bars.

As shown in Figure 3, application of ACO prior to anaerobic digestion resulted in the highest amounts of 212.1 mL/gVS biomethane from olive stones, followed by 179.7 mL/gVS and 176.2 mL/gVS for leaf and pomace samples, respectively. Figure 3-C indicates that except ACO, none

of the conducted pretreatments on olive pomace positively affected biomethane production, which may be caused by hemicellulose and oil removal during pretreatment. Although a pretreatment step could improve biomass digestibility in both AD and ethanol production processes, the choice of pretreatment method may vary depending on the target product [73]. For instance, despite *Saccharomyces cerevisiae*, AD microbes can use C-5 sugars as their carbon source, so, as shown in Figure 3, pretreatments like LHW with high hemicellulose removal has not made significant differences in methane yields of most substrates; meanwhile, ACO has improved leaves and stones' digestion through delignification, by 105% and 51%, respectively. Furthermore, anaerobic microbes are more tolerant of inhibitors like HMF and furfural, which may be produced among ACO pretreatment [73].

3.4. Scenario II: Ethanol production from olive wastes

All pretreated and untreated olive wastes have been used as feedstock for ethanol production, and separated lignin through pretreatments served as a side-product in the second scenario. Fermentation has been carried out in 118 ml bottles through the NSSF method. As presented in Figure 4, fermentation of untreated substrates resulted in 28.3%, 34.7%, and 51.6% ethanol yield for stone, both pomace and wood, and leaf samples, respectively. Given the low production yields of untreated substrates, applying a pretreatment step seems necessary. As is apparent, organosolv pretreatment caused the highest ethanol yield of 82.9% for wood samples and maximum productivity of 68.8% and 60.9% obtained through ACO pretreatment for pomace and stone, respectively. In fact, glucose inhibition and non-optimal temperature for each step are the main drawbacks of SHF and SSF, respectively, which have been resolved in the NSSF process. So that produced glucose is consumed immediately by yeast, and both enzymatic hydrolysis and fermentation process are conducted at their own optimum temperature [74]. Wu et al. [75]

revealed that a NSSF system with 10 FPU enzyme loading could achieve the same ethanol yield as an SSF process with 15 FPU enzymes, which indicates an improvement in enzymatic hydrolysis yield and confirms the obtained results in the present study. LHW has increased ethanol yield by 43% for wood samples, 59% for pomaces, and 48% for stones, while it has not made significant differences in leaf production (ANOVA).

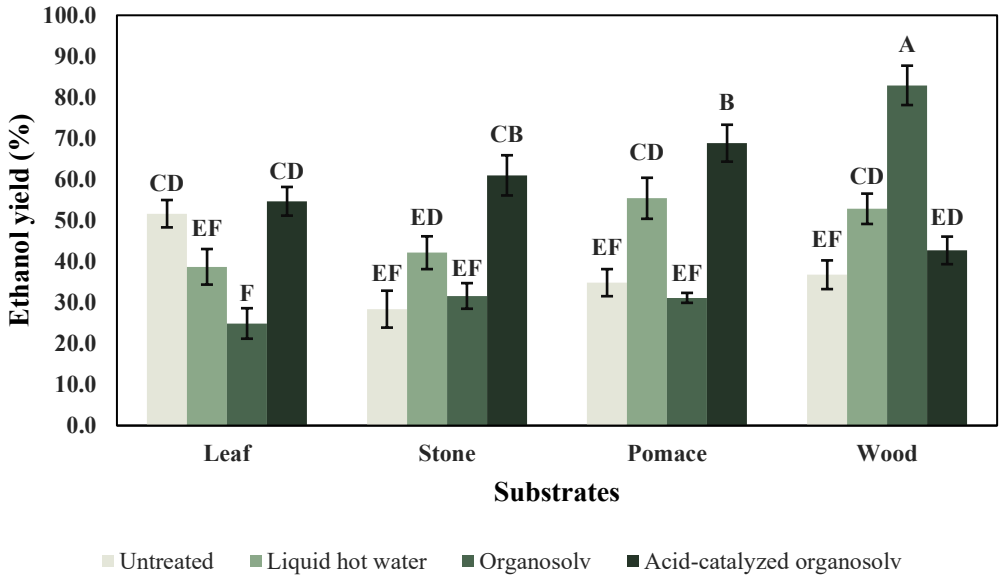


Figure 4. Ethanol production yields of untreated and pretreated samples. There is a significant difference between columns with different letters. Standard deviations are reported as error bars.

As mentioned earlier, organosolv pretreatment, with 125% yield improvement, was the most suitable method for treating wood samples prior to the fermentation process. However, it seems that an inhibition mechanism has happened in the fermentation of organosolv pretreated leaf, stone, and pomace. Compared to hydrolysis results in Figure 2, these samples have a higher potential for ethanol production than current results. Considering the complicated structure of yeast cells, there are many energy, transport, and biosynthetic pathways; besides, each inhibitor has a different metabolic effect [76]. Nevertheless, probable inhibition mechanisms are

fermentation byproducts inhibition-caused by organic acids like acetic, formic, and lactic acid, as well as a change in yeast metabolism towards the aerobic pathway, which may happen by air entrance during the sampling process. In aerobic fermentation, microorganisms take ethanol as their carbon source and reduce the final product concentration, consequently [75]. In accordance with our findings on organosolv pretreated solids' fermentation, Cuevas et al. [77] have also proved that LHW pretreated olive stone has a higher ethanol yield than organosolv pretreated ones. Inhibition tracking was not our focus in this study, but it worth more investigation. ACO has almost doubled the production yields for stone and pomace samples while it was not efficient enough for wood and leaf substrates. As mentioned in section 3.1, these two substrates contain considerable amounts of extractives (Table 1), which implementation of ACO, being exposed to the water-ethanol mixture at high temperature in acidic condition, acting as some kind of extraction process for them. Recent studies indicate that the pH of extraction assesses the character and composition of released phenolic compounds, and lower pH would cause certain interactions among phenols or degradation products like HMF and furfural [78]. So, it could explain the inhibition that occurred in the fermentation of ACO pretreated wood and leaves. Rubio et al. studied the effect of pH on phenolic compounds of olive pomace, extracted by ethyl acetate from hydrothermal pretreatment liquor, and confirmed the critical role of pH on the composition of obtained extracts.

3.5. Scenario III: A biorefinery process based on olive wastes

In the third scenario of the present study, olive wastes have been utilized for ethanol production; the produced ethanol was subsequently separated through a distillation step. Afterward, a nutrient-rich solid fraction containing yeast debris, cellulose and hemicellulose residues, lignin, and ash remained [79]. While its disposal may cause environmental concerns, the abundant

carbon and nutrient content makes it a suitable feedstock for anaerobic digestion [80]. Therefore the fermentation residues were used for biogas production, and the separated lignin through organosolv and ACO pretreatments was also considered a high-value byproduct. The amounts of obtained biomethane for each residue are presented in Figure 5.

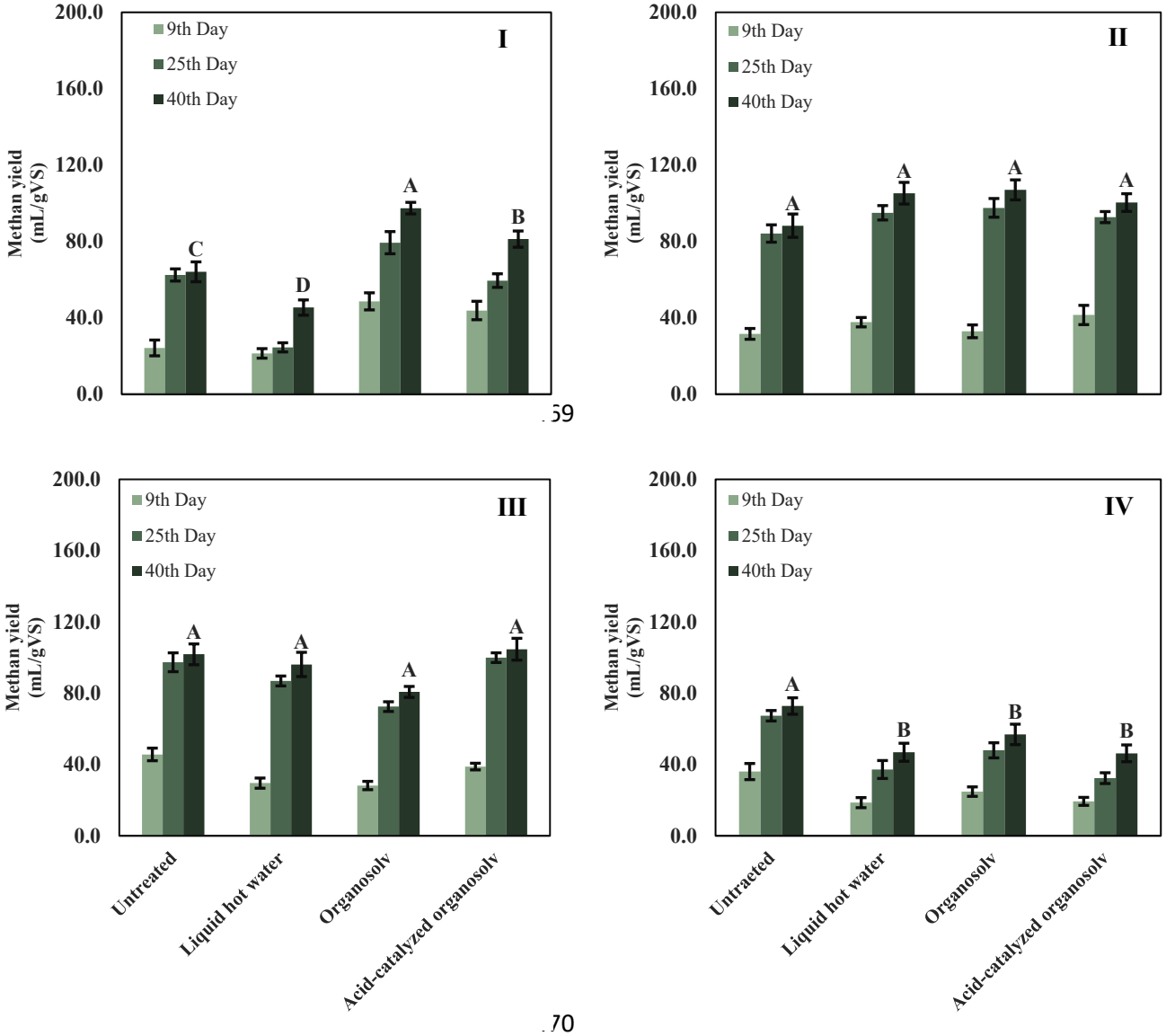


Figure 5. Methane production yields for fermentation residues of (I) leaves, (II) stone, (III) pomace, and (IV) wood after 9 days, 25 days, and 40 days anaerobic digestion. Standard deviations are reported as error bars.

As is apparent from Figure 5, there was no significant difference between the methane yield of pretreated and untreated residues for most samples. Except for the untreated leaf and organosolv pretreated wood samples with maximum production of 97.5 and 72.8 mL/gVS, the amounts of cumulative biomethane for stone and pomace were similar in different states, around average values of 100.3 and 95.8 mL/gVS, respectively. Since most of the cellulose and hemicellulose content of substrates have been used in the fermentation process or removed within the performed pretreatments, the methane yield for fermentation residues is lower than regular samples.

It is worth mentioning that even the remained digestate after the AD process is applicable as a fertilizer since it provides nutrients (N, P, K) and meliorates the soil structure with the addition of organic substances [81].

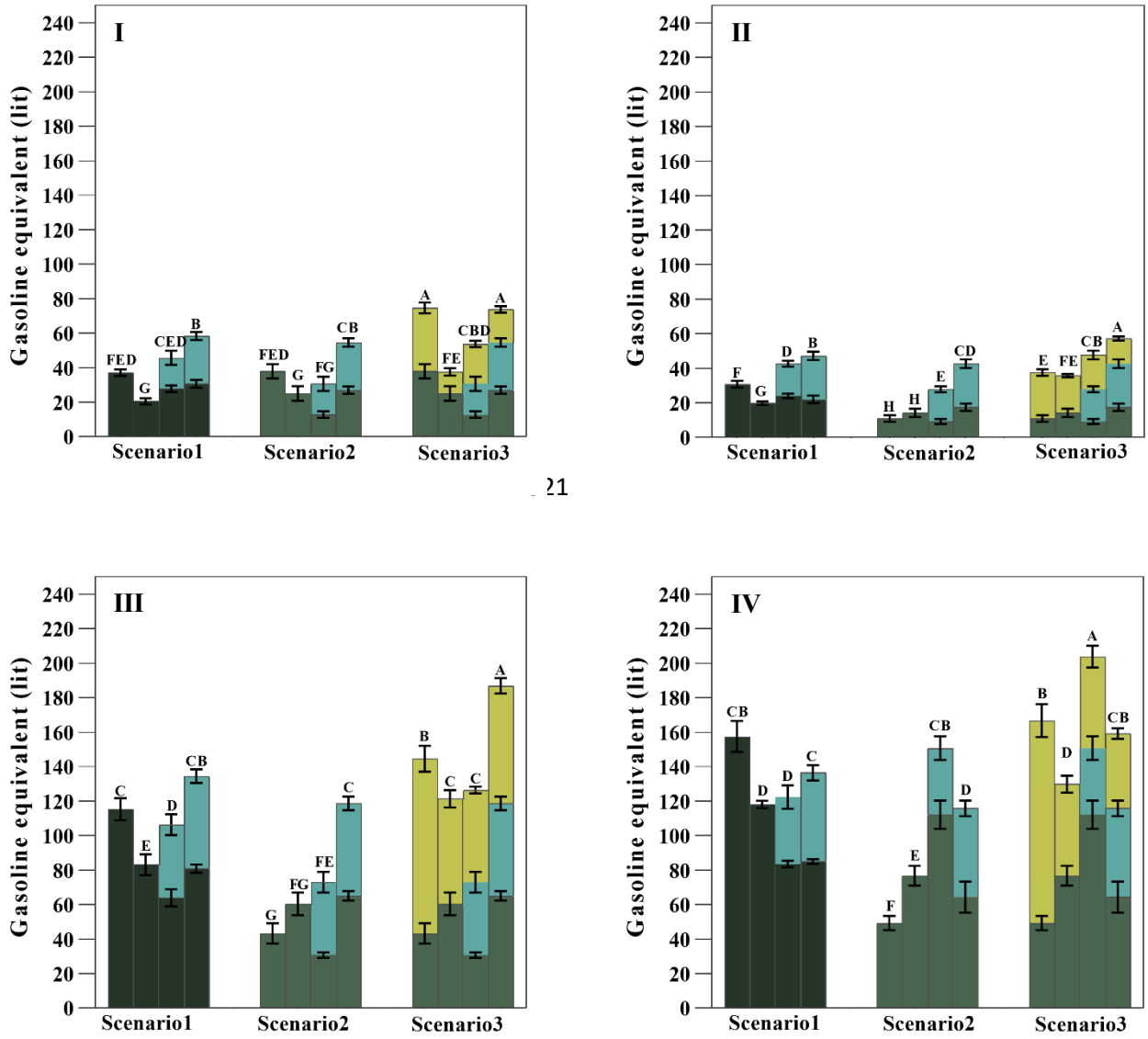
Accordingly, as a consequence of applying the third scenario, a multi-product plant by minimum waste streams was developed, which not only addressed many environmental concerns from different points of view but also improved the process economy and made it more feasible.

3.6. Gasoline equivalent

In order to evaluate the most efficient scenario for biofuel production from olive wastes, the overall energy outputs of scenarios should be compared together. Thus, the gasoline equivalent (GE) values (liter) for all kinds of produced bioenergy within the three scenarios were calculated based on 1 hectare of olive trees. Statistical information about 1 hectare of olive farms, like production quantity, amounts of pruning wastes, and value-added products, originated from the

olive fruit, have been gathered. About 56,000 hectares of land in Iran were under olive cultivation in 2019, and each hectare contains almost 180-200 olive trees resulting in 1.5 tonnes of tree pruning. The annual olive production is reported to be 109,000 tonnes (approximately 2 tonnes per hectare). As mentioned in section 2.1, the studied substrates belonged to the Zard cultivar, a kind of dual-purpose olives capable of both olive oil production and table olive preparation, which is widely cultivated in Iran [82]. To facilitate identifying the most efficient scenario for each substrate, the results were categorized into four general groups based on the substrates' type (Figure 6).

As shown in Figure 6, among the studied scenarios, ethanol production along with AD of fermentation residues (third scenario) have led to the highest GE for all types of olive wastes. ACO caused the highest energy output for both pomace and stone samples with 186.8 and 57.3 L GE through this scenario. Additionally, the organosolv pretreatment improved the wood conversions to the highest amount of 203.7 L GE. The untreated and ACO pretreated leaves with 74.7 and 73.8 L GE were the most efficient leaf samples, respectively (statistically, there was not a significant difference between mentioned values. Therefore, based on mentioned results, it is suggested to employ ACO for pretreatment of pomace, stone, and leaf samples and use organosolv pretreatment for wood samples. Undoubtedly, byproducts, like lignin and biomethane from fermentation residues, make the third scenario more advantageous than others.



519 21

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523

524 **Figure 6.** Obtained gasoline equivalent values through scenario I (biogas production), scenario II
525 (ethanol production), and scenario III (biorefinery based on olive wastes) are presented for (I)
526 leaf, (II) stone, (III) pomace, and (IV) wood samples**.** Biogas, ethanol, biogas from fermentation
527 residues, and lignin were indicated by black, green, yellow, and blue bars, respectively. Standard
528 deviations are reported as error bars.

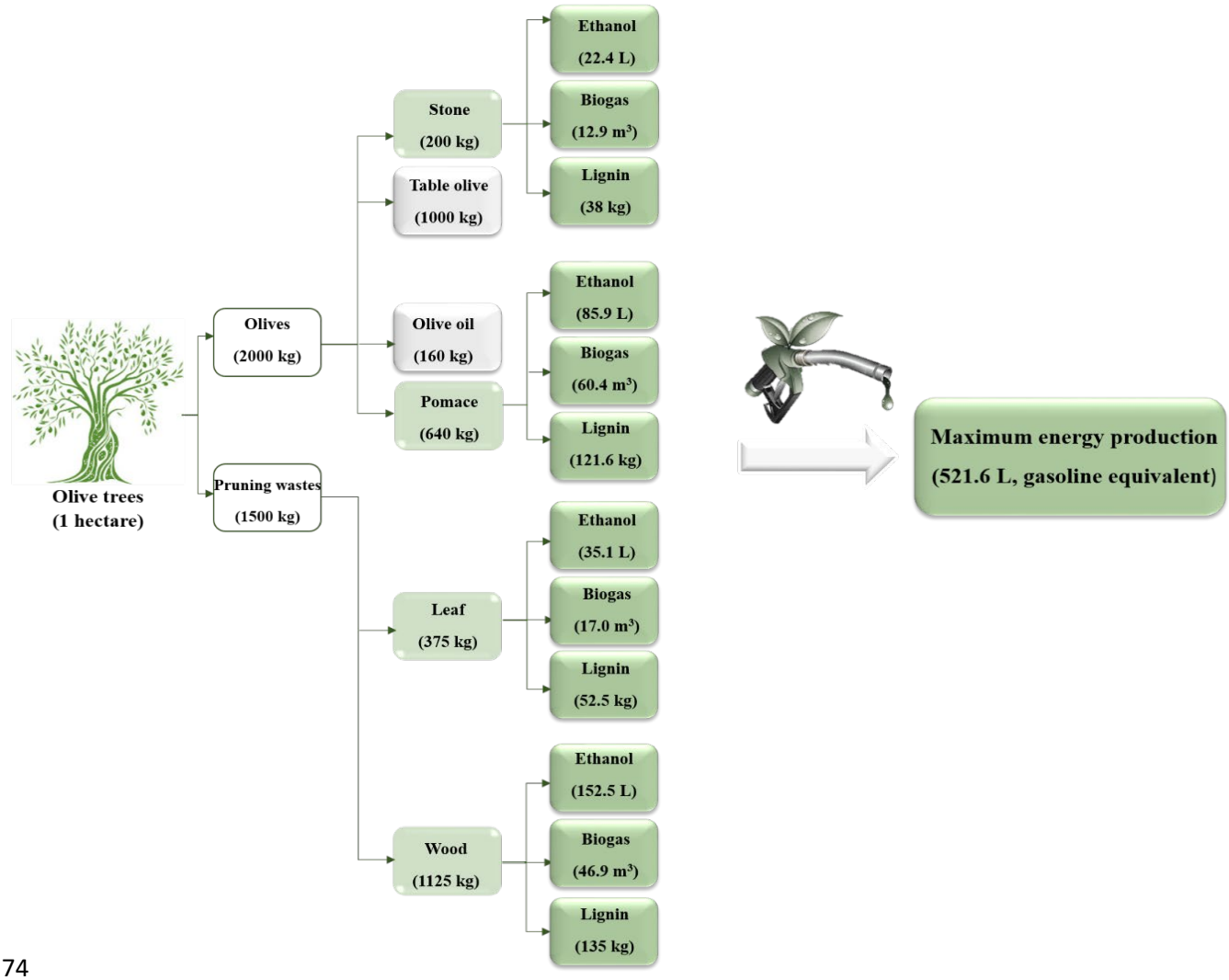
529

The HHV of separated lignins was measured through the method described in section 2.8 for all organosolv and ACO pretreated samples. The delignification yield for LHW was negligible, as discussed before. The amounts of HHV obtained as 16.8±0.3, 14.1±0.7, 21.2±0.5, 9.1±0.6 MJ/kg for leaf, pomace, stone, and wood samples, respectively. As shown in Figure 6, without lignin separation and fermentation residues digestion, the bioenergy plant would not be viable by just ethanol or biogas production. In this case, ethanol production in the second scenario resulted in GE's amounts as 12.9 and 30.7 L gasoline for leaf and pomace samples, respectively, which lignin separation increased them more than two times to 30.7 and 73 L GE, as well. Kaparaju et al. [83] studied the conversion of wheat straw to several biofuels within a biorefinery framework; they indicated that lignin would be a proficient, solid biofuel if a cheap and efficient pretreatment method or an external power plant supplies the delignification process. Moreover, the side-stream biogas has almost tripled the GE for untreated stone, pomace, and wood samples. It is worth mentioning that although the GE of untreated samples is higher than treated ones in some cases, using pretreatments still has more advantages like reducing both enzyme usage and fermenters' working volume [84]. Besides, providing value-added byproducts within the pretreatments declines capital costs and improves the process economy. The whole byproducts together have tripled the energy output of leaf samples, while the GE amounts for stone, pomace, and wood samples augmented by 5, 4.2, and 2.4 times, respectively. Therefore, a movement toward the multi-product plant is needed to make the lignocellulosic-based unit feasible and economically viable; it seems the biorefinery concept would be the best solution.

3.7. An integrated biorefinery development

Figure 7 presents a schematic overview from the outputs of our suggested biorefinery plan. The most efficient pretreated sample of each substrate, specified in the previous section, was utilized

553 through the third scenario. The amounts of produced biofuel and the gasoline equivalent of the
554 whole unit were calculated according to Eq.S1 in the supplementary data.
555 As presented in Figure 7, the whole plant is able to produce about 296 L bioethanol, and
556 anaerobic digestion of residues from the fermentation unit yields around 137.2 m^3 biomethane;
557 besides, there is a potential of lignin production for about 347.1 kg. As indicated in Figure 7, the
558 amounts of pruning waste for 1 hectare of olive trees estimated at around 1500 kg, which was
559 divided into two groups of wood (1125 kg) and leaf wastes (375 kg), and the extent of olive
560 harvesting was also predicted around 2-ton/ha. About 60% of olives are sent to table olive
561 processing plants, where the stones will be removed (200 kg), and the rest are sent to olive oil
562 production industries (640 kg wet pomace remain afterward). Therefore, wood constitutes the
563 largest proportion of olive wastes, followed by pomace, leaves, and stones, respectively.
564 Considering Figures 6 and 7, substrates' weight share has a notable role in their overall
565 production quantity. For example, despite the high potential of olive stone for both biogas and
566 bioethanol production, its least weight share among olive wastes (8%) lowers its corresponded
567 GE in a one-hectare-farm. Consequently, in an integrated biorefinery plant, all process design
568 stages, as well as pretreatment selection, should be conducted based on the characteristics of the
569 most predominant substrates. The remaining wet pomace after oil production, and the removed
570 stones from the olive fruit, still contain about 17% and 20% oil, respectively, which could be
571 extracted by a non-polar solvent like n-hexane and is potentially suitable for biodiesel production
572 [85]. The oil-free solid residues could be considered as a feedstock for biogas production to
573 expand the biorefinery plant.



574

575 **Figure 7.** An overall schematic of final products produced through suggested biorefinery plan

576 (scenario III) based on 1 hectare of olive trees.

577

578 Consequently, although olive wastes are considered lignocellulosic material in general, they are a

579 rich source of valuable components like non-edible oil and extractable phenolic compounds with

580 considerable applications in food industries as antioxidants or for medical purposes through their

581 antiseptic properties [86]. Besides, the residue output of each processing step could be subjected

582 to AD for biomethane production. Therefore a biorefinery plant based on olive wastes has

583 excellent potential for biofuel production. Moreover, techno-economical and Life-Cycle

584 Assessment (LCA) studies are needed to evaluate the whole plant from the economic and
585 environmental points of view.
586 Even though a great deal of research has been carried out on bioenergy production from
587 lignocellulosic wastes, and commercial plants in this regard have been built in the United States,
588 Brazil, Sweden, as well as Italy, most of the existing biofuels and chemicals are still derived
589 from single product chains which their feedstocks are in competition with food or feed industry
590 [87,88].

591 To promote second-generation bioenergy production, progressing research projects on biomass
592 pretreatment from lab-scale to industrial facilities is needed. In this regard, energy integration
593 should be considered in designing plants, and obtaining value from byproducts like lignin-
594 derived chemicals, antioxidants, proteins, and oils, within the concept of biorefineries, will
595 improve the economic balance of these processes [11].

596 **Conclusions**

597 Olive wastes were promising feedstocks for bioenergy production. An integrated biorefinery
598 with 295.5 L produced bioethanol, 137.2 m3 biomethane from fermentation residues, and 347.1
599 kg lignin was successfully developed. Compared to single-product plants, processing waste
600 streams to provide biomethane and lignin improved the energy recovery of the whole plant up to
601 2.5 times. ACO and organosolv pretreatments improved both ethanol and biogas production. The
602 overall produced energy per hectare of olive trees through the suggested biorefinery was equated
603 with 521.6 L gasoline. Although utilizing olive wastes for bioenergy production has several
604 beneficial aspects from the environmental and economic point of view, a techno-economic study
605 is suggested to evaluate the potential of the process for commercialization.

606 **Appendix A. Supplementary data**

Supplementary data is available in the online version.

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