Long-Term Preservation of Orange Peel Waste for the Production of Acids and Biogas

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Abstract

Orange peel waste (OPW) has a remarkable biorefinery potential. In this paper a biorefinery strategy is proposed at a laboratory-scale in order to overcome the issue of the OPW seasonality. OPW was preliminary subjected to a long-term ensiling (up to 12 weeks) with the twofold purpose of preserving the OPW potential for methane production through anaerobic digestion (AD) and stimulating the production of value-added compounds by means of biological (leachate of a previous ensiling process) and chemical (MnO_2) supplements and/or their combination. On the liquid fraction of the ensiled OPW, lactic acid (LA), acetic acid (AA) and ethanol concentrations were detected. Instead, solid fractions were used as substrates for AD both in batch and semi-continuous modes. Specifically, the combined stimulation led to LA and AA yields of 54.5 $g \cdot kg_{TS}^{-1}$ and 16.6 $g \cdot kg_{TS}^{-1}$, respectively, after 8 weeks, whereas the largest yield of ethanol (i.e., 70.4 $g \cdot kg_{TS}^{-1}$) was achieved in 8-weeks ensiling without any stimulation. Chemical and combined stimulation allow to preserve in the solid fraction, separated by centrifugation after long-term (8-12 weeks) ensiling, about 50% of the methane potential of the fresh OPW. Moreover, semi-continuous AD resulted in semi-stable processes for all the solid fractions (methane yields ranging from 0.23 to 0.28 $NL \cdot g_{VSloaded}^{-1}$) even though nutrients supplementation was necessary.

Keywords

Anaerobic digestion; Biorefinery; Ensiling; Lactic acid; Methane; Orange peel waste.

Introduction

Biorefinery facilities convert biomass constituents into marketable high-value low-volume products (e.g., organic acids, biopolymers), low-value high-volume fuels (e.g., biodiesel, bioethanol, biogas) and fertilizers [1,2]. The effectiveness of the biorefinery concept lies in the exploitation of biodegradable by-products, residues and waste generated by agriculture, industry and households thus avoiding their landfilling [3–6]. Every year, worldwide, about one-third of over 70 Mt of orange is used for industrial processing [7] and the residue produced (Orange peel waste - OPW) is widely accepted as one of the most promising feedstocks for biorefinery applications [8]. OPW (mainly composed of peel, pulp, seeds and rotten fruit) accounts for 50 – 60% (w/w) of the processed fruit [9]. Traditional routes for OPW disposal (e.g., cattle feed, composting, landfilling, incineration) are not efficient and pose several environmental issues [10] so numerous biorefinery solutions for the OPW valorisation have been explored. Indeed, OPW contains a vast variety of valuable compounds (e.g., d-limonene, pectin, enzymes, organic acids, vitamins, natural antioxidants, etc.) which can be extracted through physical-chemical methods while biological processes exploit its cellulose and hemicellulose content for bioethanol and/or biogas production [11–20]. However, the OPW biorefinery has to face some challenges: first of all, the OPW availability is seasonal so that it does not ensure a continuous year-round supply for biorefinery operations. Secondly, OPW treatments often require the use of thermal energy and chemicals or biological agents (e.g., acids, solvents, enzymes, yeasts). Furthermore, the OPW high water content and the perishable nature make its collection, transport and storage quite expensive. All these issues increase conversion processes' costs and technological complexity making most of biorefinery solutions only feasible at low processing scales [16]. In order to overcome these limitations, in this paper a new OPW biorefinery strategy has been investigated. Two subsequent biological processes have been involved: ensiling and anaerobic digestion (AD). Ensiling is a biomass preservation method routinely used in many agro-industrial installations and specifically for forage preservation. Its application is quite common and cheap (energy or materials inputs are not required) and would allow OPW availability throughout the year. During the ensiling process, autochthone microorganisms spontaneously degrade carbohydrates into valuable chemical compounds. Particularly, acetic acid bacteria (AAB) and yeasts produce acetic acid (AA) and ethanol, respectively, using sugars and residual air entrapped in the biomass. Moreover, lactic acid bacteria (LAB) convert the available substrate to lactic acid (LA) (homofermentative) and LA, acetate, ethanol and carbon dioxide (heterofermentative) when anaerobic conditions are

established [21]. As a consequence, a significant loss of biodegradable matter (expressed in terms of volatile solids, VS) is observed. Previous research reported the positive effect of ensiling stimulation by using separately i) leachate coming from a previous ensiling process containing active and already adapted microorganisms (especially LAB) and ii) manganese chloride ($MnCl_2$) [22]. Besides, LAB produce different enzymes which can be potentially utilised in different sectors. Specifically, *Leuconostoc mesenteroides*, *Leuconostoc lactis*, *Pediococcus pentosaceus*, *Lactobacillus pentosus*, and *Lactobacillus plantarum* have been reported as pectinase producers [23,24]. AD is widely accepted as one of the most convenient final valorization steps in biorefinery scenarios as it allows to effectively convert biodegradable matter into biogas (55 – 70% methane, v/v) and digestate respectively used for energy production and replacement of agricultural fertilizers [25,26]. In literature, the AD of OPW has been thoroughly reported [27–29]. Albeit scarce, information about the effect of OPW ensiling pre-treatment on AD is also available [9,28,30–32]. Unfortunately, the current literature only considers short ensiling durations and/or batch biochemical methane potential (BMP) tests have been only used to assess the AD potential. The experiment (depicted in Figure 1) consisted of a preliminary long-term OPW ensiling (up to 12 weeks) stimulated by means of biological and chemical supplements [22] and their combination (never tested before) in order to investigate the effects on the production of valuable chemical compounds (present in the liquid fraction of the ensiled OPW). Subsequently, AD batch tests were performed on the solid fractions separated by centrifugation at the end of the ensiling with the aim of determining the BMP of each individual substrate. Furthermore, semi-continuous AD reactors (one per each ensiling stimulation condition) were fed with ensiled OPW solid fractions in order to prove the feasibility of a possible full-scale application of AD using ensiled substrate along the year.

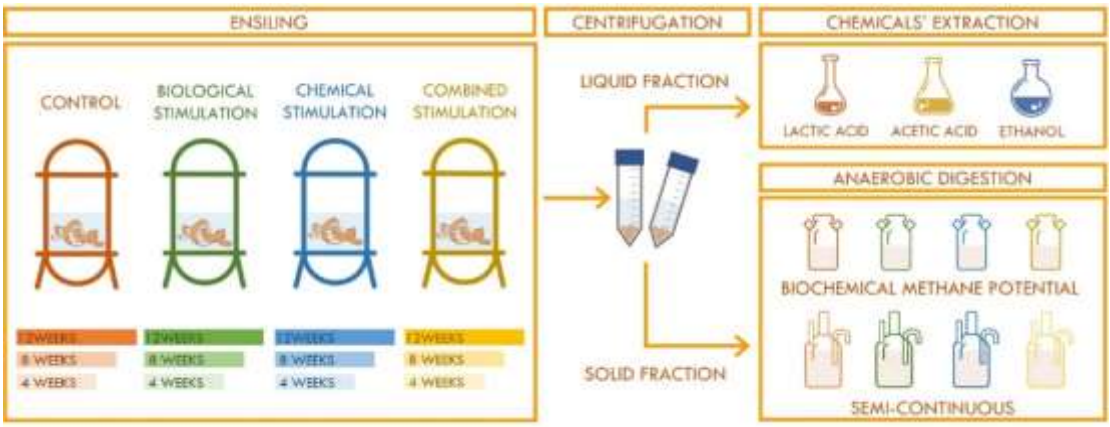

Figure 1. Experimental design

Materials and methods

The OPW used in the experiment was collected from an orange processing plant located in Reggio Calabria (Italy) and frozen at -20 °C since the freezing was not expected to affect the biological activity of the biomass [33]. Before the beginning of ensiling tests, OPW samples were thawed at room temperature.

Ensiling tests

The ensiling tests were carried out in triplicate at room temperature. They consisted of hermetically sealed glass batches loaded with about 400 g of OPW. Four different ensiling stimulation conditions were tested:

1. Control: OPW supplemented with distilled water (10% w/w).
2. Biological stimulation: OPW inoculated with a leachate produced by a previous ensiling process (10% w/w).
3. Chemical stimulation: OPW supplemented with $MnCl_2$ (at the optimal concentration of 0.005 $g \cdot kg_{OPW}^{-1}$ [22]) dissolved in distilled water (10% w/w).
4. Combined stimulation: OPW supplemented with $MnCl_2$ (concentration 0.005 $g \cdot kg_{OPW}^{-1}$) dissolved in the leachate (10% w/w). This represents the combination of the biological and chemical stimulations.

Three different ensiling durations were tested for each stimulation condition (i.e., 4, 8 and 12 weeks, respectively).

The OPW used in the ensiling tests was characterized in terms of pH, total solids (TS) and volatile solids (VS) parameters according to standard methods [34] (Table S1). At the end of the ensiling tests, the ensiled OPW was characterized in terms of pH, TS and VS in order to evaluate the changes occurred during the ensiling process. Furthermore, the ensiled OPW was subjected to centrifugation (9000 rpm for 3 minutes) in order to separate liquid and solid fraction. On the liquid fractions, LA, AA and ethanol concentrations were determined by off-line High Performance Liquid Chromatography (HPLC) analysis (Agilent 1290 infinity HPLC) equipped with an Aminex HPX-87-H column by using RID as detector according to the following parameters: mobile phase 5 mM H_2SO_4 at a speed flow of 0.6 $ml \cdot min^{-1}$ and the oven heated at 70 °C (every measurement was performed for 30 min) [35,36]. Conversely, solid fractions were

analyzed in terms of pH, TS and VS as they were used as substrates (along with fresh OPW as a control) in the subsequent AD experiments. Their characterization is reported in Table S2.

BMP tests

BMP tests were carried out according to a method extensively used in previous studies and in compliance with standardized protocols [37–39]. Four different cycles (one per each type of ensiling stimulation) were set up. Each cycle involved: i) a triplicate of the substrates (i.e., solid fractions of ensiled OPW samples) obtained from the three different ensiling times (4, 8 and 12 weeks), ii) two batches containing inoculum (in order to subtract the endogenous methane production) and iii) a triplicate loaded with fresh OPW (i.e., not ensiled) as internal control. The inoculum was collected from a full-scale anaerobic digester with a pH around 8, TS about 5% and VS about 70% of TS. The substrates were mixed with the inoculum according to a substrate to inoculum ratio (on VS basis) equal to 0.3. Besides substrate and inoculum, nutrient solutions, prepared according to the Italian standards [38] (providing anaerobic bacteria with micro- and macro-nutrients), and distilled water were also added up to reach the total working volume of 400 mL. All tests were performed under mesophilic conditions (35 ± 0.5 ° C). The produced biogas was periodically collected with a syringe and injected into an alkaline trap (NaOH 3 M) in order to evaluate the amount of methane.

Semi-Continuous AD tests

Semi-continuous AD tests were carried out using a laboratory-scale simulation system (Bioprocess Control Bioreactor, BPC Instruments) with four reactors (working volume 1.9 L) equipped with an internal stirrer and immersed in a thermostatic water bath (35 °C). This system allows the simultaneous feeding and discharge of the reactors and the produced biomethane is automatically measured by a patented system based on water/gas displacement. Each reactor was fed with the solid fraction of ensiled OPW coming from a single ensiling stimulation condition: reactor 1 for control, reactor 2 for biological stimulation, reactor 3 for chemical stimulation and reactor 4 for combined stimulation. In order to simulate real operational conditions, the substrates at different ensiling times were used in ascending order, until exhaustion of the available substrate (after 63 days of operations). Therefore, in the first phase of the tests (until day 34), 4 weeks ensiled substrates were used, in the second phase

(from day 35 to 46) 8 weeks ensiled substrates and in the third (from day 47 to 63) 12 weeks ensiled substrates were employed. The hydraulic retention time (HRT) and the organic loading rate (OLR) were set equal to 20 days and 1 $g_{VS}\cdot L^{-1}\cdot days^{-1}$, respectively. At the beginning of the tests, 5 $g\cdot L^{-1}$ of granular activated carbon (GAC, CARBOSORB 2040, 20 × 40 mesh; Comelt srl, Milan, Italy) was added to each reactor since its stabilizing effect on OPW AD has been proven [40,41]. In order to compensate the GAC lost during discharge operations, since the reactor was assumed as completely mixed, 0.48 g of GAC per day was fed throughout the entire tests' duration. Feeding was initially performed three times per week but, from day 30 onwards, substrates were added to reactors five days per week in order to make the process more stable. The pH was measured during each feeding/discharge and quantities of $NaHCO_3$ were added whenever its value was below 6.6. Moreover, on day 23 and from day 43 to 56, reactors were supplemented with the nutrient solutions used in the BMP tests to compensate the drastic reduction of ammonium measured by pre-dosed cuvette tests (Ammonium Cell Test 114.559). At the end of every week, digestate samples, collected during each feeding/discharge operations, were mixed in order to form weekly composite samples which were characterized in terms of TS, VS and volatile fatty acids (VFA) and volatile organic acids/buffering capacity ratio (FOS/TAC) through a four-point titration method [42].

Microbiological analyses

Time-course of the bacteria associated to the OPW differently ensiled (i.e., control, biological stimulation, chemical stimulation and combined stimulation) was obtained analysing the samples after 4, 8, and 12 weeks of ensiling. The bacteria load of the leachate, collected in a previous ensiling to be used as inoculum, was also considered. Briefly, each sample was ten-fold diluted, inoculated by using the spread-plate method in triplicate onto Petri plates containing de Man-Rogosa-Sharpe (MRS) agar (VWR International srl, Italy), supplemented with 15 $mg\cdot L^{-1}$ cycloheximide (Oxoid), and anaerobically incubated at 30 °C for 48 h. After counting, the colonies were randomly picked up, purified by streaking on the same isolation medium and stored as glycerol stock at 80 °C until the end of the use. The isolates were tested for catalase and Gram by KOH method [43] to select presumptive LAB. Statistical analysis was performed considering the four treatment for each sampling time. The means were analyzed

by one-way ANOVA and a Tukey's test, at 5% probability, using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

Restriction analyses and sequencing

DNA from overnight grown cultures (53 isolates), isolated from the different treatments throughout the ensiling time, was extracted by InstaGene Matrix (Bio-Rad Laboratories, USA), according to the manufacturer's instructions. Then, bacterial DNA were analyzed by PCR of the 16S rRNA gene, using the Y1 (50-TGGCTCAGAACGAACGCTGGCGGC-30) and Y2 (50-CCCACTGCTGCCTCCCGTAGGAGT-30) primers [44]. LAB were grouped by Amplified Ribosomal DNA Restriction Analysis (ARDRA) of the 16S rRNA gene using BsuRI (HaeIII) and AluI restriction enzymes (Thermo Fisher Scientific). Strains with different PCR-ARDRA profiles were chosen to sequence the 16S rRNA regions (fD1 and rD1 primers) [45]. The obtained amplicons were purified (Illustra GFX PCR DNA and Gel Band Purification Kit, GE Healthcare, UK, Limited) and sequenced by the Sanger method (Eurofins Genomics, Germany). The sequences were analyzed, compared with the sequences of the National Center for Biotechnology Information (NCBI) using the BLAST [46], and submitted to GenBank (https://submit.ncbi.nlm.nih.gov/subs/genbank/) for accession numbers. Multiplex PCR of *recA* gene was carried out to differentiate the genotypically closely related *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*), *Lactiplantibacillus pentosus* (formerly *Lactobacillus pentosus*), and *Lactiplantibacillus paraplantarum* (formerly *Lactobacillus paraplantarum*) [47].

Results and discussion

Ensiling tests

The ensiled OPW at the end of tests had similar pH in all batches (3.5 – 3.7) as previously reported [9,22,30,32]. A slight increase in pH with respect to the initial value was observed only in the case of the 12-weeks ensiling (Figure S1).

The weight loss measured during the ensiling tests was modest and rapid in all batches (2.4% at most already after the first week of operations, Figure S2) as expected [9,22]. The solid matter

reduction is widely known to be related to the degradation of OPW sugars carried out by microorganisms (i.e., AAB, LAB and yeasts) [9,31]. In terms of preservation of the biodegradable matter (hereinafter defined as "VS recovery", namely the difference between VS mass before and after the ensiling, Figure S3), biological and chemical ensiling stimulations induced VS recoveries after 4 weeks of 58% and 55%, respectively. These are slightly lower than those reported in previous studies (i.e., 64% and 58%, respectively [22]). After 8 and 12 weeks of operations, VS recoveries were lower ranging from 43% (8-weeks ensiling, chemical stimulation) to 49% (12-weeks ensiling, control). Accordingly, it can be stated that the shorter ensiling duration the lower loss of biodegradable matter. At the same time, it is evident that the increase of the loss due to the extension of the ensiling time was quite mild.

At the end of the ensiling tests, OPW samples were collected and subjected to centrifugation in order to separate liquid and solid fraction (incidence depicted in Figure S4). Indeed, liquid and solid fraction could follow different valorisation routes: the liquid could be directly used as a supplement for denitrification [48,49] or further processed to recover individual acids while the solid fraction proposed valorisation attains to the production of biogas through AD. For all tests, the amount of liquid fraction separated after long-term ensiling (i.e., 8 and 12 weeks) was similar and larger than that deriving from the 4-weeks ensiling. Particularly, biological stimulation was the condition showing the most accentuated difference (from 27.5% of liquid after 4 weeks to 50.1% and 46.7% after 8 and 12 weeks, respectively).

Table 1 reports the productivity of acids and ethanol in terms of mass of produced chemicals per kilograms of OPW (on TS basis) subjected to ensiling.

Table 1. Lactic acid, acetic acid and ethanol yields

Ensiling time	Stimulation	Lactic acid yield [g·kg$_{TS}^{-1}$]	Acetic acid yield [g·kg$_{TS}^{-1}$]	Ethanol yield [g·kg$_{TS}^{-1}$]
4 weeks	Control	21.86	7.15	44.47
	Biological	18.78	7.41	41.67
	Chemical	21.30	7.02	40.29
	Combined	20.23	8.14	35.04
8 weeks	Control	45.72	14.15	70.39
	Biological	43.44	11.16	68.11
	Chemical	38.93	12.11	61.45
	Combined	54.49	16.59	65.26
12 weeks	Control	37.53	11.52	52.37
	Biological	47.80	24.95	58.25
	Chemical	45.37	12.61	56.94
	Combined	32.72	10.79	32.34

Evidently, chemicals' yields follow the same trend of the liquid incidence (Figure S4). Indeed, the largest yields of LA and AA were achieved after 8 (for control and combined stimulation) and 12 weeks (for biological and chemical stimulations). Conversely, the ethanol productivity trend showed a peak after 8 weeks of ensiling for all tests. In previous experiments [22] authors obtained LA yields of 28.7 g·kg$_{TS}^{-1}$, 54.3 g·kg$_{TS}^{-1}$ and 55.0 g·kg$_{TS}^{-1}$ for control, biological and chemical stimulation ensiling tests, respectively, after 28 days. In light of results of these tests, it can be speculated that LA productivity of natural ensiling can be improved by extending the ensiling time (e.g., up to 8 and 12 weeks). On the other hand, it is noteworthy that only the combined stimulation, after 8 weeks of operations, reached LA yields previously determined. Referring to AA, values of AA productivity, recorded in previous research after 28 days of ensiling (i.e., 15.4 g·kg$_{TS}^{-1}$, 20.4 g·kg$_{TS}^{-1}$ and 26.1 g·kg$_{TS}^{-1}$ for control, biological and chemical stimulation ensiling tests, respectively [22]), were reached in this study only by the 12-weeks of biologically stimulated ensiling. Finally, in the case of ethanol neither the combination of stimulation conditions nor the duration of ensiling improved the yields previously determined after 28 days of ensiling (74.1 – 104.5 g·kg$_{TS}^{-1}$) [22]. To sum up, it can be concluded that longer ensiling times (up to 12 weeks) were more beneficial than stimulation in terms of value-added compounds' productions.

BMP tests

Table 2 reports the results of the BMP tests carried out on the ensiled OPW solid fractions. Given these, the comparison between the AD of 1 kg of raw OPW (i.e., without any pre-treatments) and the AD of the solid fractions separated by centrifugation after the ensiling of the same initial amount of OPW was considered in order to evaluate the incidence of the ensiling on the BMP.

Table 2. Comparison among measured BMPs, methane yields and theoretical values yielded by the AD of raw OPW

Stimulation	Ensiling time	BMP [$NL \cdot kg_{VS}^{-1}$]	St. Dev. [$NL \cdot kg_{VS}^{-1}$]	Methane yield [$NL \cdot kg_{rawOPW}^{-1}$]	$CH_4/CH_{4rawOPW}$
Not ensiled	-	429.8*	30.70*	86.6*	100.0%
Control	4 weeks	533.5	33.35	57.5	66.4%
	8 weeks	555.3	8.12	40.9	47.3%
	12 weeks	436.2	67.98	37.8	43.6%
Biological	4 weeks	553.1	37.25	61.1	70.5%
	8 weeks	462.2	21.46	34.2	39.5%
	12 weeks	459.2	21.11	36.2	41.8%
Chemical	4 weeks	555.1	39.04	56.7	65.4%
	8 weeks	525.9	47.09	39.6	45.7%
	12 weeks	565.0	23.82	45.0	52.0%
Combined	4 weeks	551.7	36.42	58.1	65.5%
	8 weeks	529.6	46.52	43.6	49.1%
	12 weeks	501.0	24.04	41.8	47.1%

*Average of 12 batches with different inocula (internal control)

In general, in spite of substrates' very low pH, all processes run regularly and no acidification occurred in any reactor due to the buffering capacity given by the inoculum and the nutrient solutions. The BMP of fresh OPW determined as average of 12 batches (used as internal control) is consistent with the literature [27]. All ensiling processes improved the conversion of OPW to methane since the ultimate methane volumes produced by the AD of the ensiled OPW substrates were higher than that determined from the fresh OPW AD. This behaviour is attributable to the effects of the ensiling also visually evident. Expressly, the OPW became an homogenous slurry already after 10 – 20 days of ensiling [9]. This increases the availability and the digestibility of the substrate to anaerobic microorganisms. It appears that the ensiling acts as a pre-treatment in which hydrolysis of the macro-molecules takes place before the following AD. The same occurrence was observed in previous studies [9] while in others [30,31] the raw OPW exhibits a larger methane potential with respect to the ensiled one. In these tests solid fractions (after centrifugation) of ensiled OPW samples were used. Thus, it can be possible that inhibitory

agents, especially limonene, were concentrated in the liquid fractions. Indeed, as several studies so far stated [9,31,50], degradation, occurring during ensiling, provokes the rupture of sacs of the peel containing limonene allowing its release. In terms of methane production, it seems that chemical and combined ensiling performed slightly better. The influence of the duration of the ensiling on methane conversion potential is not certain but 4 weeks ensiling seem to show higher BMP.

The loss of biodegradable matter (VS) and the separation of the liquid phase (accounting slightly less than 50% of the ensiled OPW and rich in sugars and biodegradable acids) was partially counterbalanced by the higher BMP of the solid fraction separated by centrifugation. In fact, especially if combined and chemical stimulation processes are adopted, it is still possible to preserve in the solid fraction, separated by centrifugation, about 50% of the original methane potential of the whole mass of fresh OPW after a very long-term ensiling. Compared to previous tests [9], the efficiency of the combined process (i.e. 4 weeks ensiling, centrifugation and AD) in terms of methane recovery is basically confirmed.

Semi-continuous AD tests

Solid fractions (after centrifugation) of the ensiled OPW materials were used as substrates for four semi-continuous AD tests (one per ensiling stimulation condition). Figure 2 depicts the daily methane productions (a) and the methane yields (b) of reactors designed as 1, 2, 3 and 4 on the basis of ensiling stimulations (i.e., control, biological, chemical and combined, respectively).

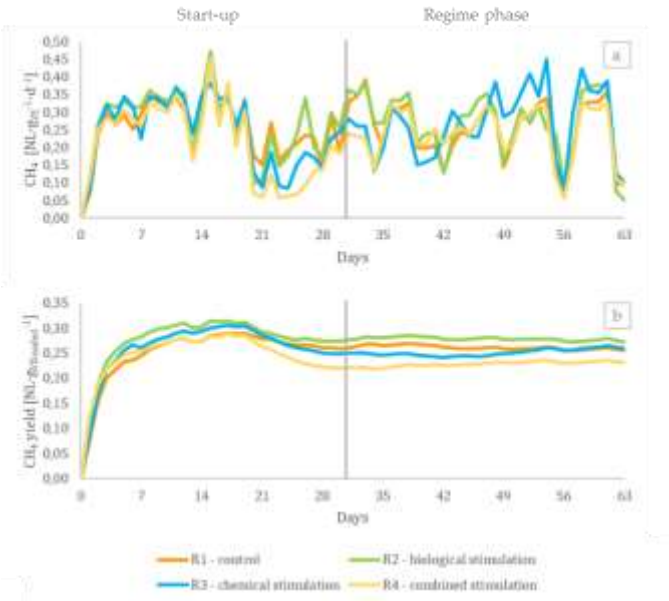

Figure 2. Daily methane production (a), methane yield (b)

In general, all reactors behaved similarly throughout the tests' time. After an acclimation phase that lasted about one week, all reactors reached their peaks of methane production on day 15 (0.38 – 0.47 $NL \cdot g_{VS}^{-1} \cdot d^{-1}$). The first 30 days of operations represented the start-up phase of the tests. Then, because of a change of the feeding procedure making processes more stable (see below for more details), the regime phase began. In terms of methane yield, the average values of the start-up phase were 0.25 $NL \cdot g_{VSloaded}^{-1}$, 0.28 $NL \cdot g_{VSloaded}^{-1}$, 0.26 $NL \cdot g_{VSloaded}^{-1}$, 0.25 $NL \cdot g_{VSloaded}^{-1}$ for reactors 1, 2, 3 and 4, respectively. During the start-up, specifically from day 21, methane productions slowed down due to pH reduction (see Figure 3, a) with minimums of 0.15 $NL \cdot g_{VS}^{-1} \cdot d^{-1}$, 0.15 $NL \cdot g_{VS}^{-1} \cdot d^{-1}$, 0.09 $NL \cdot g_{VS}^{-1} \cdot d^{-1}$, 0.06 $NL \cdot g_{VS}^{-1} \cdot d^{-1}$ for reactors 1, 2, 3 and 4, respectively, on day 23. Afterwards, production trends progressively increased again and they remained almost constant until the end of the tests. In the regime phase (i.e., from day 30 onwards), reactors exhibited average methane yields of 0.26 $NL \cdot g_{VSloaded}^{-1}$, 0.28 $NL \cdot g_{VSloaded}^{-1}$, 0.25 $NL \cdot g_{VSloaded}^{-1}$, 0.23 $NL \cdot g_{VSloaded}^{-1}$, respectively. Methane productions' variations can be properly explained through the analysis of pH, FOS/TAC, VFA and NH_4-N trends (Figure 3).

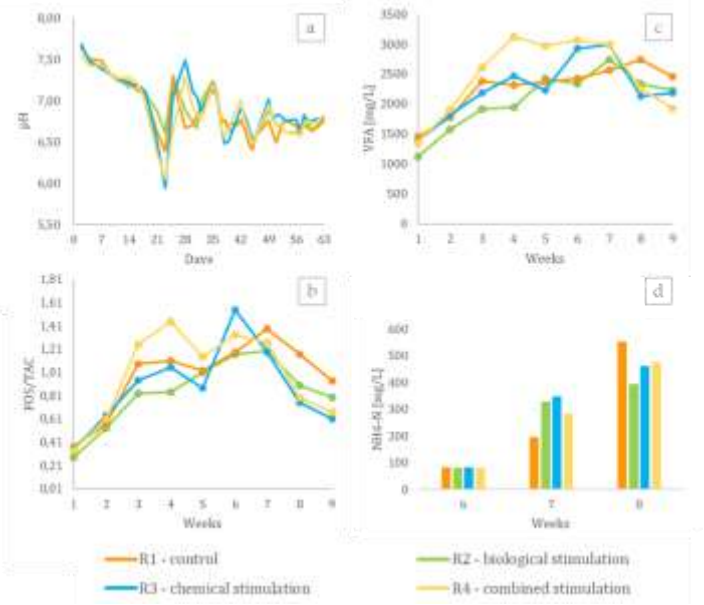

Figure 3. Semi-continuous AD tests' results: pH (a), (b) FOS/TAC (b), VFA (c), NH_4-N (d)

On day 21 acidic conditions, witnessed by the increase in VFA concentrations, were detected in all reactors (Figure 3, c). Specifically, the amount of VFA was below 1500 mg·L^{-1} at the beginning of the experiments and then it sharply increased to 2430 mg·L^{-1}, 2372 mg·L^{-1}, 2469 mg·L^{-1} and 3122 mg·L^{-1} in reactors 1, 2, 3 and 4, respectively, between the fourth and fifth week. The excessive accumulation of organic acids is evident in FOS/TAC trends dramatically increasing up to the fourth week (Figure 3, b). This points out that the buffering capacity of the systems was not adequate to balance the growing presence of acids generated by the acidogenic bacteria that were not converted to methane due to the different effect of methanogenic and acidogenic bacteria in the substrate kinetic [51]. Moreover, this situation could have been exacerbated in this case since hydrolysis of the macro-molecules of the OPW probably already took place during the ensiling step, as previously argued, so that the substrate was promptly available for acidogens. As a consequence, pH in all reactors clearly showed downward trends with serious drops on day 23 (pH of 6.61, 6.39, 5.94, 6.05 for reactors 1, 2, 3 and 4, respectively) (Figure 3, a). In order to restore favourable conditions, the feeding was suspended on days 23 and 24 and 2 g of $NaHCO_3$ were added in all reactors. Then, from day 30 onwards, the amount of substrate (calculated on the basis of the fixed OLR, i.e., 1 g_{VS}·L^{-1}·d^{-1}) was fed to reactors five days per week instead of three times per week as carried out previously. The AD process improved with this change in the feeding procedure as witnessed by the respective daily methane production and pH growing trends. The brief feeding suspension allowed methanogens to digest the extra quantity of VFA in the first place. Secondly, the change in the feeding pattern probably improved

the synergistic interaction between acidogens and methanogens. Besides, lack of nutrients represented another serious issue for the semi-continuous processes. OPW is widely known to be a nitrogen-poor substrate and therefore techniques (such as co-digestion or nutrient solutions supplementation [41,52]) are often adopted to reach the optimal carbon to nitrogen ratio. From the analyses of ammonium concentration, carried out on week 6 on discharged digestates, it clearly emerged that all reactors suffered from too low nitrogen presence (less than 100 $mg \cdot L^{-1}$) (Figure 3, d). This occurrence probably impaired acidogenesis and methanogenesis again (see peaks in FOS/TAC values on weeks 6 and 7, Figure 3, b). For this reason, from day 43 to 56 the amount of water necessary to dilute input substrates was replaced with an equal volume of nutrient solutions [38]. This practice was suspended when NH_4-N concentration in all reactors exceeded the threshold of 200 $mg \cdot L^{-1}$ which is widely beneficial to anaerobic processes [53]. Thanks to these two adjustments, all processes proceeded stably until the exhaustion of input substrates.

Referring to the type of input substrates, reactor 4 (fed with the solid fraction of the ensiled OPW subjected to combined stimulation) more severely suffered from acidosis than the others as witnessed by larger values of FOS/TAC and VFA concentration (Figures 3, b and c). Accordingly, its recovery occurred slower than the other reactors (Figure 2). However, differences in average methane productions were lower than 20% as methane yields ranged from 0.23 to 0.28 $NL \cdot g_{VSloaded}^{-1}$ in the regime phase. Also, the feeding of materials coming from the three different ensiling times (i.e., 4, 8 and 12 weeks) seems not to have affected processes as no relevant variations in parameters were detected after changing input substrates (on days 35 and 47). In terms of methane generation, methane yields detected in these tests are consistent with those determined previously (i.e., ranging from 0.20 to 0.25 $NL \cdot g_{VSloaded}^{-1}$) [40,41]. However, it is also clear that the lack of nitrogen represents an important issue and that co-digestion with a substrate with a high buffering capacity and rich in nitrogen (e.g., cow manure) would be ideal for the process.

Microbiological changes

The loads refer to the pre-ensiling leachates and the OPW differently ensiled analysed throughout the ensiling time. Concerning the pre-ensiling leachate, the bacteria were in the range 4.98 – 6.72 Log UFC/mL that could be due to the environmental temperature variation occurring during the massive production of the leachate to inoculate all the experiments.

Throughout the ensiling weeks, the population was almost stable in the control treatment while it increased in the biological and chemical treatments after 8 weeks and in the mixed stimulation after 12 weeks reaching the highest value of 7.71 Log UFC/mL (Figure S5). This could be attributable to the stimulating role of the $MnCl_2$ on LAB [54–57]. Anyway, it is to highlight the moderate load variation among the different ensiling treatments.

LABs' identification and specie distribution

85% of the isolated bacteria was catalase negative and Gram positive while the remaining 15% was catalase positive and Gram negative, not further considered in this study. Six patterns of ARDRA profiles were observed (data not shown). LABs were identified as *Lactiplantibacillus plantarum*, *Lactiplantibacillus paraplantarum*, *Levilactobacillus brevis* (formerly *Lactobacillus brevis*), *Paucilactobacillus suebicus* (formerly *Lactobacillus suebicus*), *Schleiferilactobacillus harbinensis* (formerly *Lactobacillus harbinensis*), and *Lentilactobacillus* sp. Considering the percentage of identity obtained by BLAST for *Lentilactobacillus* sp., further analyses are necessary to assign it either to *Lentilactobacillus otakiensis* or to *Lentilactibacillus sunkii*. The accession numbers of the LAB strains sequenced and deposited to GenBank are: OM980227 for OPB 2 *L. plantarum* (% similarity 99% - accession of the closest relative by BLAST NR_104573.1), OM980228 for OPB 4 *L. brevis* (% similarity 99% - accession of the closest relative by BLAST NR_044704.2), OM980229 for OPB 15 *P. suebicus* (% similarity 99% - accession of the closest relative by BLAST NR_114977.1), and OM980230 for OPB 45 *S. harbinensis* (% similarity 99% - accession of the closest relative by BLAST NR_113969.1).

Figure 4 reports the LAB species frequency of the different trials at each stage of the ensiling.

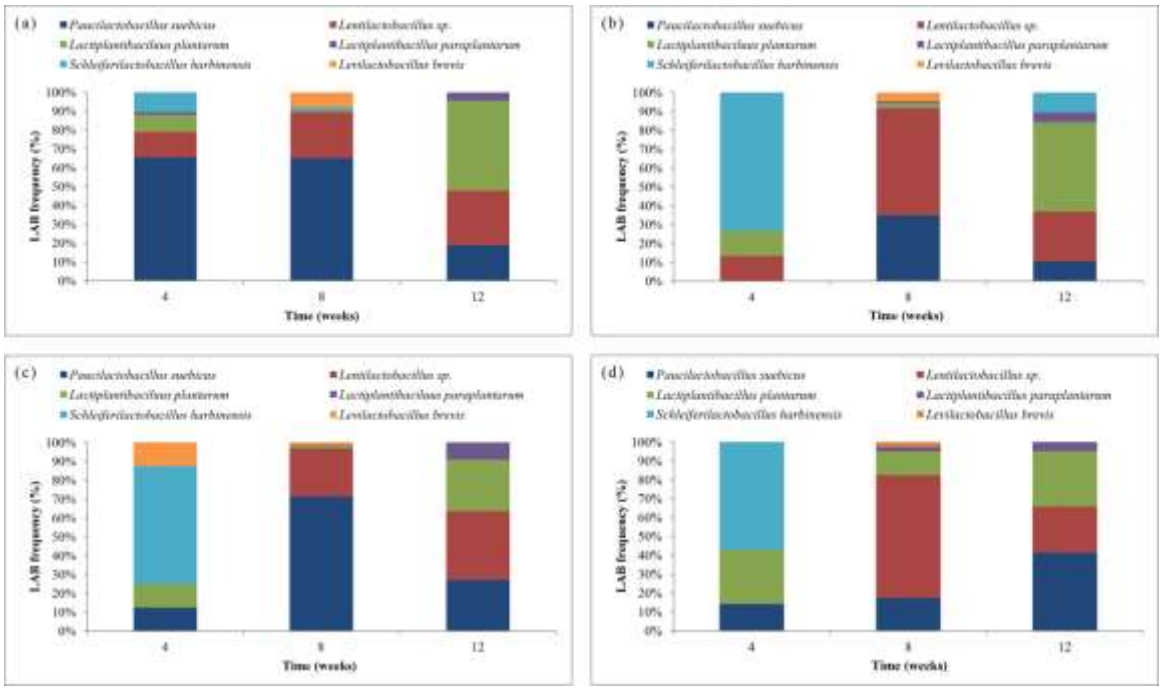

Figure 4. LAB specie frequency, expressed as percentage, detected at each stage of the different ensiling treatments: control (a), biological (b), chemical (c) and combined (d).

The species isolated from the pre-ensiling leachates were *L. plantarum*, *P. suebicus*, *S. harbinensis*; therefore, the other species detected in the ensiling treatments were associated to the raw OPW used in the experiments. Compared to the control (A), where the *P. suebicus* was dominant up to 8 weeks while after 12 weeks *L. plantarum* became dominant, at the start of the ensiling all the treatments favoured the growth of *S. harbinensis*. After 8 weeks of ensiling, the biological and the comnbined treatments allowed the growth of *Lentilactobacillus* sp. becoming the dominant specie. At the end of the ensiling, all the treatments except the biological one showed the same species composition. Anyway, the *L. plantarum* were dominant in A and B treatments and it was the second as percentage of frequency after *Lentilactobacillus* sp. and *P. suebicus* in C and D treatments, respectively. The isolated LAB specie are coherent with the matrix used since they are typical of fruits and fermented vegetables [9,58–60]. In particular, *L. plantarum* is considered the most useful homofermentative LAB for improving the quality of the silage fermentation [61–63].

Conclusion

In this paper the first attempt to simulate at a laboratory-scale a new biorefinery strategy for OPW management is presented. The OPW was preliminary ensiled at different durations (i.e., 4, 8 and 12 weeks) and different stimulation conditions (i.e., biological, chemical and their

combination). In summary, it resulted that the long-term ensiling induced greater organic matter loss but an improvement of LA and AA content (in the liquid fractions) compared to shorter ensiling durations. Subsequently, the solid fractions of ensiled OPW were used as substrates in AD tests. BMPs determined in batch tests ranged from 436.2 to 555.3 $NL \cdot kg_{VS}^{-1}$ while semi-continuous AD tests showed methane yields ranging from 0.23 to 0.28 $NL \cdot g_{VSloaded}^{-1}$ even though processes suffered from lack of nutrients (e.g., nitrogen). Finally, it can be stated that the presented biorefinery solution would allow i) to make OPW available throughout the year, ii) to recover a liquid fraction directly employable either for valuable chemicals' extraction or carbon supplement for biological denitrification of wastewater and iii) to generate methane and digestate from the anaerobic digestion of the residual solid fraction. Further research is advisable in order to investigate the performance of the anaerobic digestion of the whole long-term ensiled OPW material (i.e., without separating liquid and solid fractions) and its co-digestion with and N-rich substrate.

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