Integral valorization of orange peel waste through optimized ensiling: Lactic acid and bioethanol production

Integral valorisation of orange peel waste through optimized ensiling: lactic acid and bioethanol production

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Abstract

The management of the huge amount of orange peel waste (OPW) is a complex issue although it has a very high potential in terms of biorefining. One of the main problems in the valorisation of OPW is the seasonality of its production with the ensiling method being largely proposed as a possible solution . During the ensiling process, value added chemicals including lactic acid, acetic acid and ethanol are spontaneously produced together with a significant loss of volatile solids (VS) . In this contribution, the stimulation of lactic acid bacteria by either a biological (inoculation with leachate coming from a previous ensiling process) or chemical ($MnCl_2$ supplementation) methods has been tested with the aim to increase the chemicals production preventing, at the same time, the VS loss. The inoculation with the leachate improves both the VS recovery (+7%) and the concentration of lactic acid (+113%) with respect to the uninoculated one (control). The overall yields of the process are noticeable, up to about 55 $g \cdot kg_{TS}^{-1}$ of lactic acid, 26 $g \cdot kg_{TS}^{-1}$ of acetic acid and 120 g· g·kgTS-1 of ethanol have been produced. On the other hand, the chemical stimulation enhances the production of liquid products together with a significant VS loss. The proposed preservation method, due to its

simplicity, can be easily implemented at full-scale allowing the production of added-value chemicals and the concurrent storage of the OPW that can be further valorised (e.g. animal feed, pectin or biomethane production).

Keywords: Acetic acid; ensiling; ethanol; lactic acid; microbial population; orange peel waste

Highlights

- Full valorisation of orange peel waste (OPW) is highly advantageous
- Ensiling is a suitable preservation method for OPW prior to valorisation
- Ensiling can be optimized for preserving as many volatile solids as possible
- During the ensiling process, lactic acid and other chemicals can be efficiently produced

1. Introduction

The management of the huge amount of orange peel waste (OPW), produced each year worldwide as a by-product of orange juice manufacturing (de la Torre et al., 2019; United States Department of Agriculture - Foreign Agricultural Service, 2020) is a very complex issue.

At the moment, most of the produced OPW is dumped near production sites or in landfills causing severe impacts on air, soil and water while its use as animal feed, although quite common, is limited by difficulties related also to transportation costs (Ahmadi et al., 2015; Ricci et al., 2019; Zema, 2017; Zema et al., 2012).

The biorefining potential of this residue is very high since the extraction of value-added products such as chemicals, bioactive compounds for the preparation of nutraceuticals, sorbents for water treatment and bio-combustibles has been fully demonstrated at industrial, pilot or laboratory scale (Mahato et al., 2020; Satari and Karimi, 2018; Sharma et al., 2017; Zema et

al., 2018). On the other hand, some issues still remain: the large amount produced, the seasonality and the presence of residual d-Limonene (toxic for microorganism and thus problematic in case of biologic valorisation processes) prevent the full exploitation of the OPW (Ángel Siles López et al., 2010; Benito et al., 2016; Moraci et al., 2011; Tian et al., 2014; Zema et al., 2018).

The ensiling process (a forage preservation method under anaerobic conditions) has proven to be a suitable option to overcome these limitations since it can preserve the biomass reducing, at the same time, d-limonene content thus allowing its use along the year (Ashbell and Lisker, 1987; Büyükkılıç Beyzi et al., 2018; Calabrò et al., 2020; P. S. Calabrò et al., 2018; Calabrò and Panzera, 2018). One of the key limits of the ensiling process is the considerable loss of volatile solids (VS) that can reach up to 40% of the initial mass.

During the ensiling, a spontaneous anaerobic fermentation process occurs without the need of any particular inoculation or management operation leading to the production of several chemical compounds. In particular, lactic acid bacteria (LAB) produce lactic acid (homofermentative) together with acetate, ethanol and carbon dioxide (heterofermentative). In particular, yeasts utilise sugars for the production of ethanol while acetic acid bacteria (AAB) oxidise sugars and ethanol using the residual air entrapped in the biomass during the silo loading (Liang et al., 2016; Mamlouk and Gullo, 2013). The valorisation of the OPW by spontaneous or inoculated ensiling is a research field in expansion (Calabrò et al., 2019; Ricci et al., 2019; Zerva et al., 2019). However, more research effort is needed to optimize the process without a significant increase of the overall complexity/cost of the ensiling (e.g. use of specialized microorganisms, complex management operations such as pH or temperature control). In this context, in order to improve both the VS preservation as well as the production of value-added chemicals (with particular attention to lactic acid), the stimulation of LAB is a possibility to be explored. This stimulation could be implemented by either the inoculation of already adapted

76 microorganisms contained in the leachate of a previous ensiling experiment or by the addition
77 of manganese chloride due to its stimulating role on LAB (Archibald, 1986; Ohkouchi and
78 Inoue, 2006; Racchach and Marshall, 1985; Terpstra et al., 2001).
79 Even if the catalytic valorisation of lignocellulosic waste and residues into energy, fuel and
80 chemicals is a well consolidated field of research (Luque and Clark, 2013; Xu et al., 2020),
81 research on valorisation of OPW through the ensiling procedure for the production of high
82 added-value feedstocks (lactic acid especially) for modern biorefineries is lacking in the
83 literature.
84 This research activity has two main objectives: (i) to reduce the VS loss occurring during the
85 ensiling in view of the possible valorisation of OPW (e.g. use as animal feed, pectin production,
86 anaerobic digestion) and (ii) to increase the production of high added-value compounds (e.g.
87 ethanol and lactic acid).
88 A strong advantage of the proposed OPW valorisation process is the simplicity of the adopted
89 approach, that avoids pH and temperature control and the use of specialized microbial strains.
90

91 ## 2. Materials and Methods

92 ### *2.1 Ensiling process*

93 The OPW used for the ensiling tests was collected from an orange processing industry in Reggio
94 Calabria – Italy (Agrumaria Reggina s.r.l.). After sampling, the OWP was frozen at – 20 °C
95 (freezing is not expected to affect its biological activity (Pognani et al., 2012)). Samples were
96 then thawed at room temperature and the OPW was characterized in terms of pH, TS and VS
97 parameters, according to APHA et al. (2012), before the beginning of the experiment.

98 The experiment procedure (Figure 1) consisted of two stages. First, 750 g of OPW were equally
99 divided and ensiled in three glass batches in order to produce a leachate that was used as
100 inoculum for the subsequent experiment. Each batch was hermetically sealed by a stopper and

connected to an acidic trap (pH<5) to allow the biogas venting. After 28 days, the ensiling was stopped and the OPW was extracted and centrifuged at 9000 rpm for 3 min in order to separate the liquid fraction.

In the second part of the experiment (see Figure 1), five ensiling modes were tested:

1. the blank (control) sample (OPW supplemented with distilled water - 10% w/w);
2. the OPW inoculated with the leachate (10% w/w) produced in the previous experiment;
3. the OPW supplemented with $MnCl_2$, at a dosage of 0.05 g/kg_{OPW} and dissolved in distilled water (10% w/w);
4. the OPW supplemented with $MnCl_2$, at a dosage of 0.01 g/kg_{OPW} and dissolved in distilled water (10% w/w);
5. the OPW supplemented with $MnCl_2$, at a dosage of 0.005 g/kg_{OPW} and dissolved in distilled water (10% w/w).

Each ensiling test was carried out in duplicate using 400 g of OPW per batch and lasted 28 days (Calabrò et al., 2020; P. S. Calabrò et al., 2018; Calabrò and Panzera, 2017). The biogas volume (> 95% CO_2 - Calabrò et al., 2020) was measured by the water displacement method using an acidic trap. The biogas produced during the ensiling process flowed into another reactor filled with an acidic solution (the trap), the pressure increase displaced a volume of solution equal to that of the biogas produced that was then collected into a graduated cylinder where it was measured(Gou et al., 2014).

The determination of pH, TS, VS (APHA et al., 2012) was done on the ensiled samples collected both after 10 and 28 days. Moreover, in addition to the analyses mentioned above, the quantitative analysis of lactic acid, acetic acid and ethanol products in the liquid phase separated from those samples by centrifugation was also carried out. In particular, the analysis of VS was used for mass balances in order to calculate the recovery respectively after ensiling (the amount

present in the sample respect to that fed at the beginning of the process) and in the solid phase separated by centrifugation.

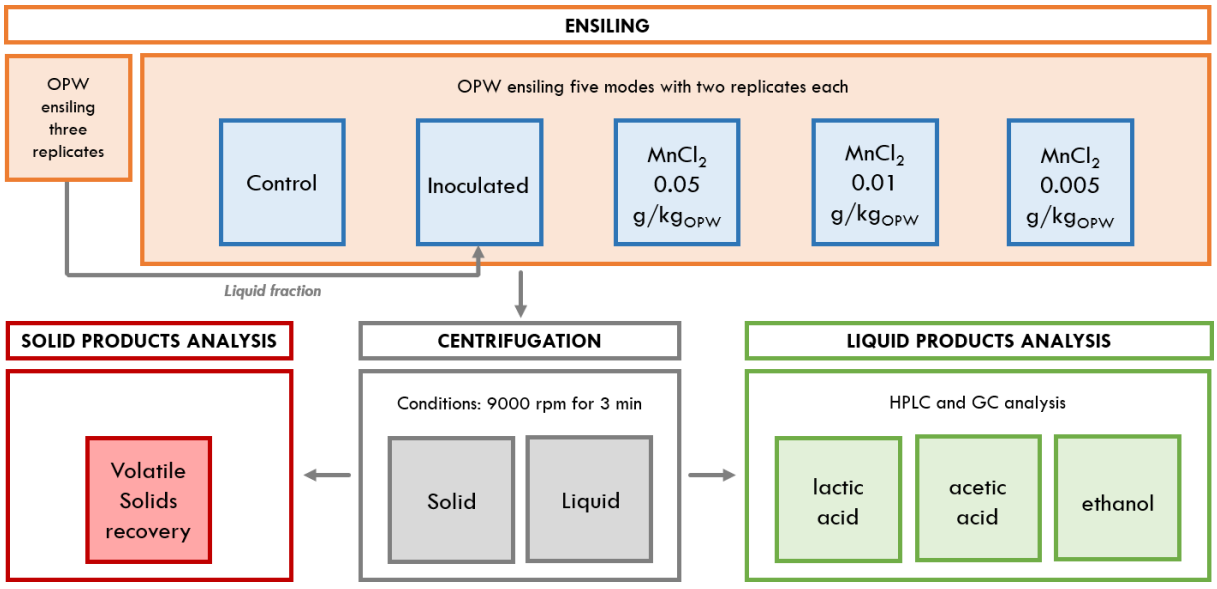


Figure 1. Experimental design for the valorisation of orange peel waste through optimized ensiling

2.2 Analytical methods

The quantitative determination of lactic acid, acetic acid and ethanol was carried out following an analytical procedure reported in the reported in the last years by some of the authors (Gumina et al., 2019; Malara et al., 2018). Products present in the aqueous liquid phase were quantified through an 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, following these parameters: mobile phase 5mM H_2SO_4 at a speed flow of 0,6 ml/min and the oven heated at 70°C (every measurement was performed for 30 min).

Products in the liquid phase were also cross-checked by GC analysis (organic compounds were previously extracted with ethyl acetate and eventual water traces were removed by adding anhydrous sodium sulphate to the organic phase) by using an off-line gas chromatograph

(Agilent 6890 N) equipped with a wide-bore capillary column (CP-WAX 52CB, 60 m, i.d. 0.53 mm) and a flame ionization detector (FID). The injector was settled at 250 ◦C and the temperature program started from 50 °C (held for 5 min) up to 240 °C with a 10 °C/min rate (held for 10 min) and finally at 240 ◦C (held for 5 min) during the post run (Paone et al., 2020).

2.3 Statistical analysis

A one-way ANalysis Of VAriance (ANOVA) with repeated measures at the sampling dates of the batch experiments was carried out to check the significance of differences in biogas production, VS loss and production of chemicals during the ensiling process (the batch type was assumed as an independent factor). Dunnett's test was used to check if the statistical differences ($p < 0.05$) between control and the other treatments in terms of yield of ethanol, lactic and acetic acid are significant.

ANOVA assumes the normal distribution of residuals. This assumption was checked using the Adersen-Darling method, based on the function of the empirical distribution. All residuals of data resulted normally distributed ($p < 0.05$).

The XLSTAT software release 1.2019 was used for all statistical analysis.

3. Results

Table 1 shows the characteristics of the OPW used as feedstock for the experiments.

Table 1. Main characteristic of raw OPW

Parameter	**Average Value**	**St. Dev.**
pH	4,10	-
TS [%]	18,5%	0,66%
VS [%TS]	95,9%	0,39%

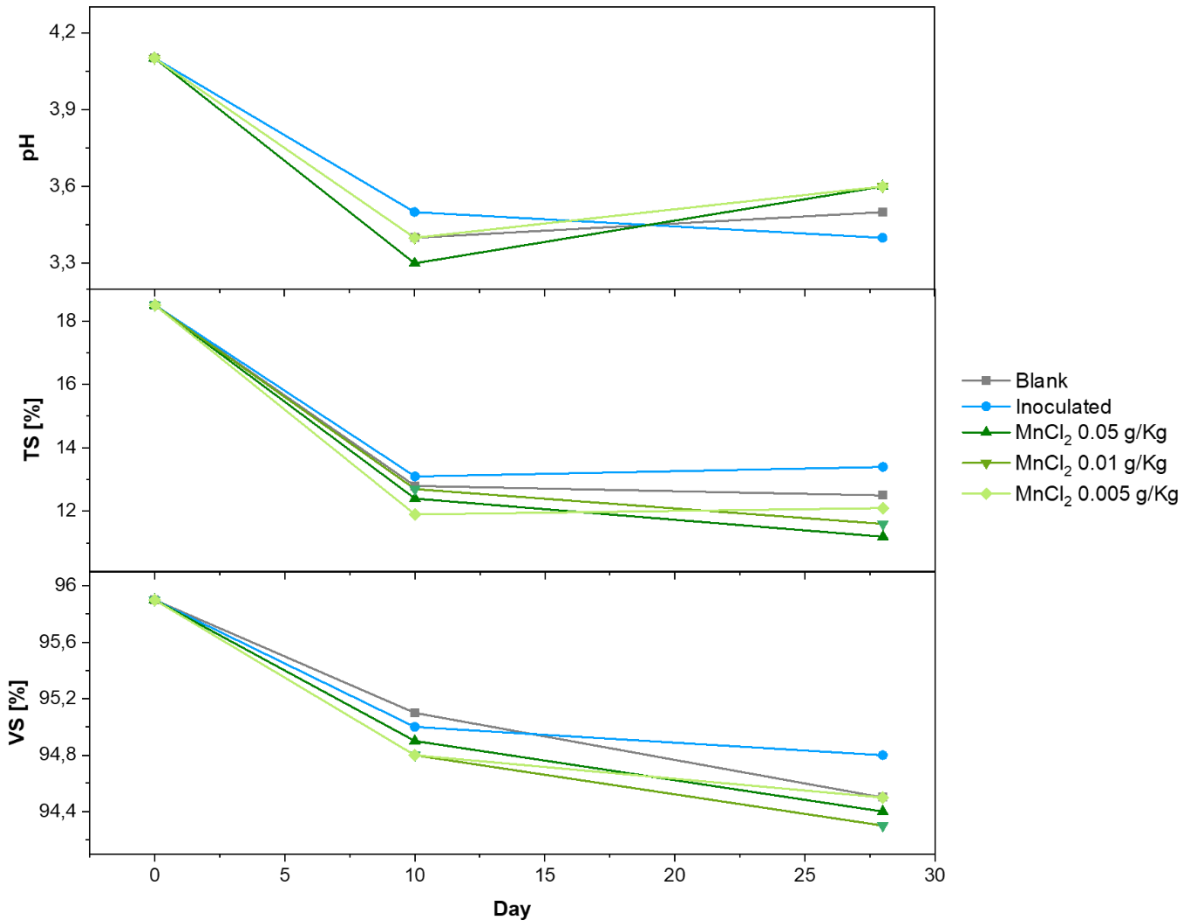


Figure 2. pH, Total Solids (TS) and Volatile Solids (VS) trends during the ensiling processes

During the ensiling process, pH values show a rapid decrease from the initial value of 4.1 to about 3.4 followed by a slight increase for all samples excepted those coming from inoculated batches (Figure 2). At the same time, total solids (TS) significantly decrease in the first 10 days (Figure 2) while a certain stability is evidenced for all the treatments in the final part of the experiment excepted those supplemented with the highest amounts of $MnCl_2$. Accordingly, VS trend is continuously decreasing but the reduction is larger in the first 10 days (Figure 2). In days 10-28, in the inoculated reactor, VS consumption is slower than that observed in the others while at the end of the process the reactor exhibited the highest VS value.

The biogas production (Figure 3) seems to follow two different patterns. The amount of biogas produced during the experiment is more regular in blank and inoculated batches: it is

concentrated in the first 10 days similarly to those of the two replicates of the same ensiling mode (differences from the average of the cumulated production from the single batch are limited to less than 20%). However, in the inoculated batch, the cumulated biogas production is 30% higher than that observed in blank samples (where biogas production is completed in 13-16 days).

In manganese chloride supplemented batches, the production of biogas is lower and irregular (differences between the replicates are larger). In these experiments, biogas production lasts 4-9 days and it seems to be inversely correlated to the amount of $MnCl_2$ supplemented: by increasing the total addition of $MnCl_2$ a lower biogas production is observed.

Noticeably, ANOVA ($p<0.05$) did not show any statistically significant difference among treatments.

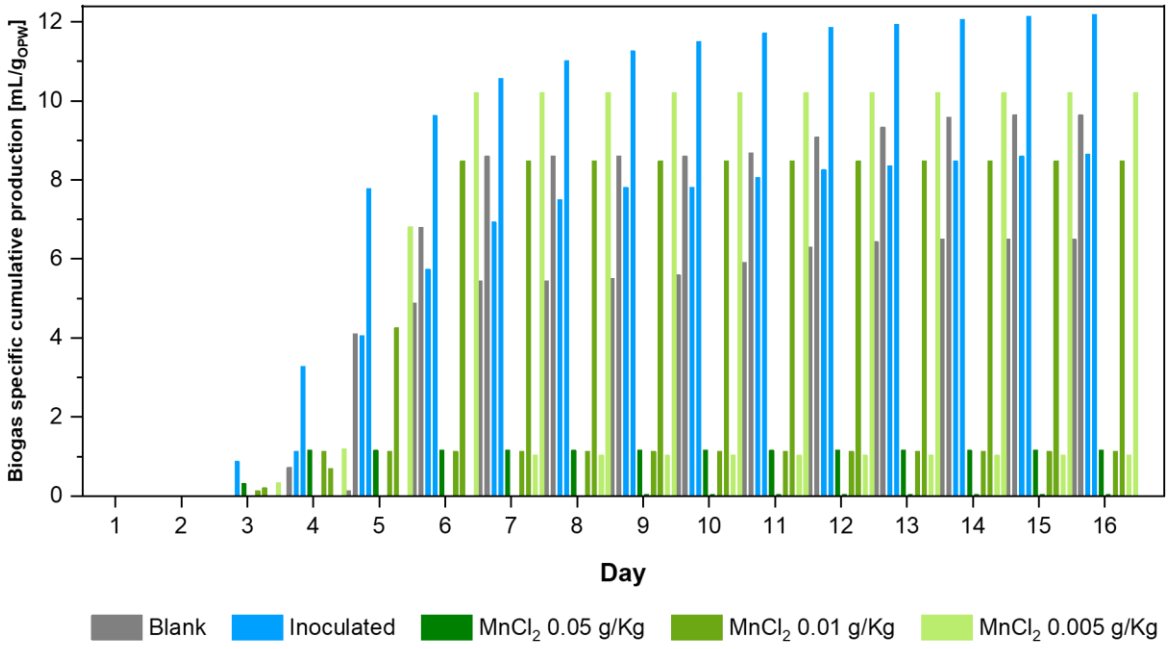


Figure 3. Biogas specific cumulative production during the ensiling processes.

The mass balance depicted in Figure 4 demonstrates that the VS loss during the ensiling occurs mainly (69 – 88% of the total) in the first 10 days when , the biological activity is more intense

as witnessed also by TS, VS and biogas production trends. The total loss accounts for 35 – 47% of the VS initially present in the batch. In the reactors supplemented with $MnCl_2$, the VS loss is higher and continues in the second part of the ensiling experiment (days 10-28).

Also in this case, ANOVA ($p<0.05$) does not show any statistically significant difference among treatments.

Figure 5 further confirms these trends: the liquid fraction - separated by centrifugation - is larger in manganese chloride supplemented batches most probably as a consequence of the longer and more intense biological activity.

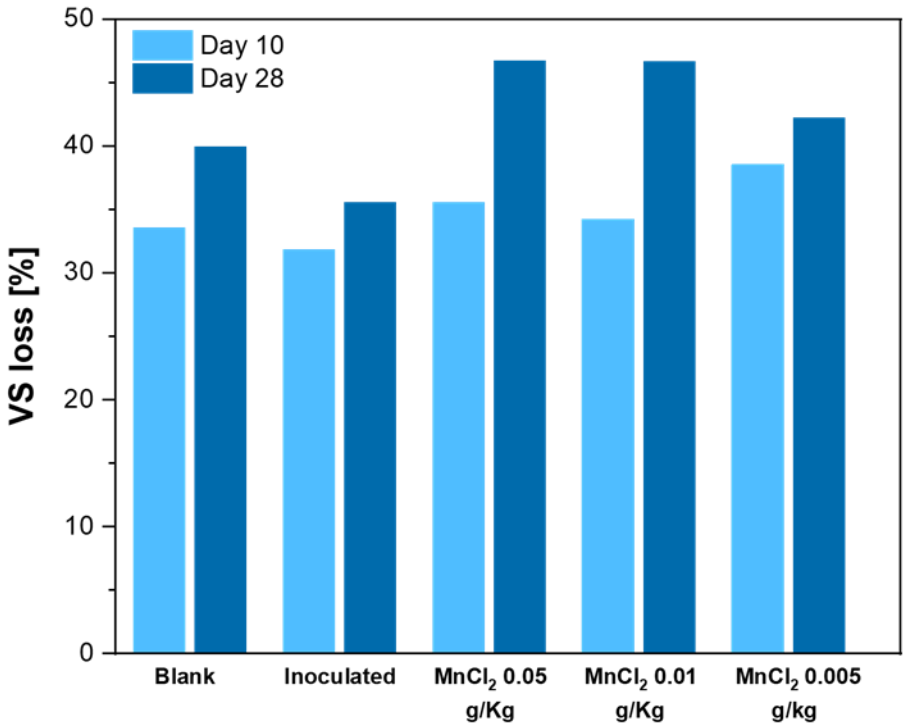
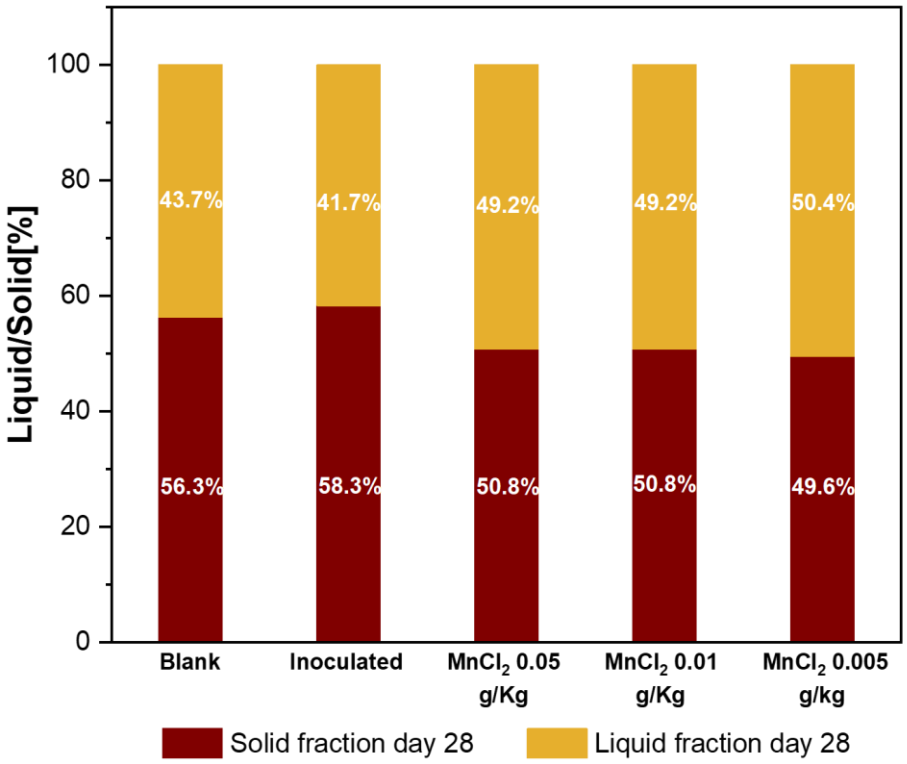


Figure 4. Volatile solids loss during the ensiling processes.



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205 **Figure 5.** Incidence of the solid and liquid fraction at the end of the ensiling processes.

206

207 **Table 2.** Volatile solids recovery after the ensiling process (28 days) and in the solid fraction,

208 separated by centrifugation, with respect to the VS initially present

Batch	Ensiling mode	VS Recovery after ensiling		VS recovery by centrifugation (solid fraction)	
		Replicates	Average	Replicates	Average
1	Blank	60,5%	60,1%	39,6%	41,4%
2		59,6%		43,1%	
3	Inoculated	68,0%	64,5%	46,9%	46,8%
4		61,0%		46,7%	
5	$MnCl_2$ 0.05 g/kg	50,1%	53,3%	37,6%	37,5%
6		56,5%		37,4%	
7	$MnCl_2$ 0.01 g/kg	54,9%	55,4%	38,2%	38,2%
8		55,9%		38,2%	
9	$MnCl_2$ 0.005 g/kg	59,3%	57,8%	37,5%	37,2%
10		56,2%		37,0%	

Indeed, Table 2 outlines how the VS recovery in the solid fraction separated by centrifugation is larger (+23-26%) in inoculated batches with respect to manganese chloride supplemented reactors.

Furthermore, at the day 10, acids concentrations (especially lactic acid)are largely higher in the inoculated reactor with respect to the others, as witnessed also by the statistical analysis (Table 3). This trend is partially confirmed, in the case of lactic acid, by experiments at day 28. In this case, $MnCl_2$ supplemented reactors performed quite well (Table 4). It is interesting to highlight that the lactic acid concentration detected at both 10 and 28 days is higher on using the manganese chloride supplemented reactors compared to the blank ones. Acetic acid concentrations, measured at the end of experiments, are more uniform. For what ethanol is concerned, concentrations are similar in all the batches both at day 10 and 28, as also outlined by statistical analysis.

Table 3. Ethanol, Lactic and Acetic acid concentration in the separated liquid at day 10. The same superscript letter denotes statistical similarity at $p<0.05$.

Day 10	**Lactic acid [g/L]**		**Acetic acid [g/L]**		**Ethanol [g/L]**	
Ensiling condition	**Replicates**	**Average**	**Replicates**	**Average**	**Replicates**	**Average**
Blank	2.16 1.64	1.90^{A}	1.23 1.18	1.21^{A}	26.19 26.53	26.36^{A}
Inoculated	13.50 13.67	13.59^{B}	5.65 5.69	5.67^{B}	22.79 24.91	23.85^{A}
$MnCl_2$ 0.05 g/kg	1.90 2.16	2.03^{A}	1.30 1.53	1.42^{A}	-* 25.61	25.61^{A}
$MnCl_2$ 0.01 g/kg	2.13 3.41	2.77^{A}	2.21 2.10	2.16^{C}	25.51 27.30	26.41^{A}
$MnCl_2$ 0.005 g/kg	3.03 2.33	2.68^{A}	2.28 2.19	2.24^{C}	27.26 24.97	26.12^{A}

*outlier value

Table 4. Ethanol, Lactic and Acetic acid concentration in the separated liquid at day 28. The same superscript letter denotes statistical similarity at $p<0.05$.

Day 28	Lactic acid [g/L]		Acetic acid [g/L]		Ethanol [g/L]	
Ensiling condition	**Replicates**	**Average**	**Replicates**	**Average**	**Replicates**	**Average**
Blank	8.72 8.20	8.46^{A}	4.58 4.53	4.56^{A}	26.32 26.42	26.37^{A}
Inoculated	17.90 18.15	18.03^{B}	6.87 6.67	6.77^{B}	23.25 25.95	24.60^{A}
$MnCl_2$ 0.05 g/kg	12.36 12.11	12.24^{C}	6.25 5.94	6.10^{C}	-* 27.53	27.53^{A}
$MnCl_2$ 0.01 g/kg	12.92 13.69	13.31^{C}	6.51 6.39	$6.45^{B,C}$	26.84 25.51	26.18^{A}
$MnCl_2$ 0.005 g/kg	14.23 13.17	13.70^{C}	6.40 6.59	$6.50^{B,C}$	26.37 25.67	26.02^{A}

*outlier value

Table 5. Ethanol, Lactic and Acetic acid yields (day 28).

Day 28	**Lactic acid [g kg_{TS}^{-1}]**	**Acetic acid [g kg_{TS}^{-1}]**	**Ethanol [g kg_{TS}^{-1}]**
Blank	28.7±2.6	15.4±1.0	89.2±5.4
Inoculated	54.3±0.1*	20.4±0.4*	74.1±3.7
$MnCl_2$ 0.05 g/kg	51.8±0.4*	25.8±0.6*	117.0±0.0
$MnCl_2$ 0.01 g/kg	54.3±1.5*	26.3±0.3*	106.8±2.9
$MnCl_2$ 0.005 g/kg	55.0±2.0*	26.1±0.4*	104.5±1.1

* Indicate a statistical difference respect to control ($p<0.05$)

Yields from TS added at the beginning of the ensiling (Table 5) demonstrate the clear superiority of inoculated and $MnCl_2$ supplemented batches in terms of lactic and acetic acids production. In the case of ethanol, blank and inoculated batches show a similar behaviour while those chemically stimulated give a better result. Dunnett test confirms that the performance of the inoculated and manganese supplemented batches is statistically different from blank.

4. Discussion

Data on TS, VS and biogas production demonstrate that the stabilization of the OPW for blank and inoculated reactors occurs quickly, in agreement with previous reports(Calabrò et al., 2020; Calabrò and Panzera, 2018). The process is characterised by a significant biogas production that stops in about 10-15 days. The difference in biogas production observed between the blank and the inoculated reactors confirms that the microbial consortium already adapted to the OPW rapidly and intensely guides the process. Manganese chloride supplemented reactors behave differently, the VS consumption (witnessed by the steeper decreasing trend in days 10-28) continues for a longer period but the total biogas production is significantly lower. These findings makes possible to hypothesize that the manganese chloride supplementation induces changes in the microbial consortium and/or in its metabolism leading to a reduced CO_2 production. This hypothesis is supported if we take into account that $MnCl_2$ was reported to modify the bacterial community composition during the anaerobic digestion (Cai et al., 2018) affecting homofermentative and heterofermentative LAB (Raccach, 1985) and influencing, at the same time the CO_2 production.

With respect to the declared objectives of this paper, it seems that the biological stimulation, induced by the use of the already evolved microbial inoculum (contained in the liquid coming from a previous ensiling process) is beneficial for a faster stabilization of OPW with a concurrent better preservation of the VS (Table 2). Infact, as expected, the blank and the inoculated reactors exhibit a similar trend.This was predictable since the liquid used to inoculate the reactors derives from the ensiling of the same OPW matrix used for the blank reactors. Clearly, the lower values observed for the blank reactors indicates how the autochthonous microbial population needs time to stabilize and to evolvie while it is already established in the inoculated reactors. These results confirm the advantage of bioaugmentation in the ensiling similarly to the anaerobic digestion process (Calabrò et al., 2018; Yu et al., 2016).

The more prolonged and intense use of VS in manganese chloride supplemented reactors is coherent with a lower recovery of VS at the end of the ensiling and after centrifugation (Table 2).

The addition of the leachate, coming from a previous ensiling cycle does not significantly influence the concentrations of lactic and acetic acids at day 10, since the volume of liquid present in the batch is about four times higher than that added at day 0. By means of a mass balance, it is possible to calculate that the leachate added at day 0 (whose composition was assumed equal to that of the leachate collected at the end of the ensiling in the blank reactor) contains 3% of the lactic acid, 5% of the acetic acid and 26% of the ethanol found at day 10.

This confirms that the production of acids in the first 10 days in the inoculated reactor is fast and larger than in the other reactors. In the case of lactic acid (Table 4), at day 28, its concentration in the inoculated reactor is 113% higher than that of the blank, while in manganese chloride supplemented reactors the concentration is increased to a lower extend (+45/+62%) with respect to the blank. It is worth to underline that a minor amount of $MnCl_2$ has a positive effect on the lactic acid production. The better performance of inoculated reactors with respect to the blank is related to the rapid degradation activity of the naturally evolved microbial consortium originated from previous OPW ensiling (Sivagurunathan et al., 2016; Yan et al., 2012). The improved lactic acid production in manganese chloride supplemented reactors compared to the blank ones confirms the positive effect of the inorganic salt on the growth and metabolic activities of LAB (Raccach, 1985) even if an excessive concentration has toxic effects (Nsair et al., 2020) and this justifies the better results obtained with more limited supplementation of $MnCl_2$. However, it must be pointed out that if the lactic acid yield is considered instead of its concentration, , the production is similar in inoculated and chemically supplemented reactors (the higher amount of liquid fraction available in manganese supplemented reactors needs to be taken into account). The uniformity of the amount of ethanol

found in reactors containing different treatments as well as in the two sampling times (day 10 and day 28) supports the idea that its is suppressed very early, before day 10. It is plausible that the autochthonous yeasts population (mainly ethanol producers) behave similarly to that analysed during ensiling experiments carried out by some of the authors on analogous samples. It was shown that this population reaches the maximum load after seven days of the ensiling and then gradually decreases (Calabrò et al., 2020). Moreover, it seems that the yeasts inhabiting fresh OPW are, to some extent, naturally resistant to the D-limonene with differentiation of species throughout the ensilage time. In this context, it has been already reported (Calabrò et al., 2020; Wilkins et al., 2007) the ethanol production from the citrus peel waste using *Saccharomyces cerevisiae* and enzymes such as β-glucosidase that hydrolyse polysaccharides into sugars ready to be used by yeasts. Engineered *S. cerevisiae* for β-glucosidase, able to use cellobiose, have also been reported (Adam and Polaina, 1991; Tokuhiro et al., 2008). Considering that yeasts belonging to different species naturally occurring in vegetal material can possess, at different rates, the β-glucosidase activity (Sidari et al., 2019), it cannot be excluded that the autochthonous species of yeast, inhabiting the OPW, contribute to the process by saccharification and/or fermentation.

At the best of the authors' knowledge, there is only one paper in the scientific literature that allows a partial comparison with the results of this research. For what the lactic acid yield is concerned (Ricci et al., 2019), used rewetted OPW for a solid state fermentation at a laboratory scale (5 g of dry substrate used in each replicate) by selected microbial strains, in pH and temperature controlled conditions, and obtained a maximum of 209.65 g kg^{-1}. Although higher, this production is fully comparable with those of the experiments presented in this paper (Table 5) obtained without the need of a specialized inoculum, pH and temperature control with the obvious benefits in terms of process economy and simplicity.

5. Conclusions

This research outlines how it is possible to produce high added-value chemicals such as lactic acid without any significant modification of the currently applied procedure for the ensiling process.

In particular, the inoculation with the leachate produced during the ensiling improved both VS recovery (+7%) and concentration of lactic acid (+113%) with respect to the uninoculated one (blank).

The yields of the process are noticeable, up to about 55 $g \cdot kg_{TS}^{-1}$ of lactic acid, 26 $g \cdot kg_{TS}^{-1}$ of acetic acid and 120 $g \cdot kg_{TS}^{-1}$ of ethanol have been produced.

The chemical stimulation carried out supplementing manganese chloride, enhances the production of value-added chemicals during the ensiling process but also the VS loss. Experiments on the long-term effects of the manganese chloride supplementation are necessary for a more comprehensive evaluation and for the optimization of the process as a function of the maximization of the most desired output (i.e. feedstock production or VS preservation).

Additionl research experiments are currently ongoing on the valorisation of the solid OPW, obtained by centrifugation, and on the analysis the effect of a prolonged ensiling on the yields of the process as well as on the possibility of combining chemical and biological stimulation.

The practical implications of this research are very promising since its full-scale implementation would allow, at the same time, to produce value-added chemicals during the ensiling and to store the OPW for further valorisations processes (e.g. animal feed, pectin or biomethane production).

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