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Lactic Acid production from Tomato Pomace Fermentable Sugars using Innovative Biological Treatments

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The organic residues from agro-food sector can be transformed into valuable economic resources rather than representing an unmanageable waste, whose disposal in landfills contributes to increase both soil pollution and the greenhouse effect. With this in mind, the aim of the investigation is to design eco-friendly compatible innovative biological processes for producing lactic acid (LA) from fermentable sugars using as substrate tomato pomace. In the first part of the investigation, we show the physico-chemical characterization of tomato pomace produced in the 2017 season with or without thermal pre-treatment. pH, total and volatile solids content, sugars and cellulose are measured. The microorganism consortia suitable for lactic acid production are selected from buffalo dung, and compared with commercial strains of lactic acid producing bacteria. Both bacteria consortia are used as inoculum for the anaerobic fermentation of tomato pomace as sole substrate for producing fermentable sugars and convert them in LA. Sugars are measured by a spectrophotometric-coupled enzyme assay and LA by HPLC with electrical conductivity detector. The molecular analysis of the isolated bacteria is performed by using the denaturing gradient gel electrophoresis (DGGE). Anaerobic fermentation of tomato pomace is also performed using different initial pH values of the substrate. The final goal is to design biological processes aimed at achieving the highest LA recovery yields.

1. Introduction

Italy has produced over the past three years about 5 million tonnes per year of fresh tomatoes for processing industries, providing 48% of European tomato processed products (ANSA, 2016). Tomato pomace (TP), which is the by-product of tomato processing, accounts for about 4.5 % of the whole tomato. TP is the mixture of tomato peels, crushed seeds and small amounts of pulp that remain after the processing of the tomato for juice, paste and ketchup (Heuzé et al., 2015). Only in Campania region, during the 2015 warm-season about 290 kt of fresh tomatoes were processed producing 13 kt of tomato residues, of which the dry fraction, constitutes around 4 kt (Brachi, 2016). TP is a low-cost substrate made for about 65% of lignocellulosic matter on dry basis (Cepeda and Collado, 2014). It is currently used as low value livestock feed or mostly inexpensively disposed directly on the soil as fertilizers. However, TP rots very quickly, causing water pollution, unpleasant smells and greenhouse effect due to release of large amounts of carbon dioxide and methane in the atmosphere (Mangut et al., 2006). Nonetheless, such wastes can be upgraded into valuable organic acids by fermentation processes, in particular sugars can be converted into lactic acid (LA), which holds an important position because of its several applications in food, pharmaceutical, cosmetics, textiles as well as the production of biodegradable poly-lactic acid (PLA) (Panesar and Kaur, 2015). In fact, TP is composed of a heterogeneous polysaccharidic mix (pectins, cellulose and hemicelluloses) associated with components like lignin, which could be used as raw materials in anaerobic digestion, potentially using indigenous bacteria present in animal manure. Efficient utilization of both cellulose and hemicellulose-derived sugars can reduce the cost of production of biobased chemicals by as much as 25% (Hinman et al.,1989). A

key step in using these sugars is the degradation of cell walls into sugar monomers, a process called saccharification. Mechanical pre-treatments as milling, grinding, or cutting reduce biomass particle size, and increase the susceptibility of biomass to enzymatic attack and enzymatic hydrolysis to release constituent sugars from biomass (Howard et al., 2003).
Organic acids, such as LA, are formed during the breakdown of the biomasses by acidogens microbial populations. They represent the intermediate products of the anaerobic fermentation, whose final products are carbon dioxide and methane when the process time is extended. Therefore, the aim of the present investigation is to test innovative eco-friendly biological processes for producing LA from tomato pomace fermentable sugars using bacteria consortia obtained from water buffalo manure in comparison with the most efficient commercial LA producing bacteria, as complementary process to biogas production.

2. Materials and Methods

The present study was carried out between October 2017 and January 2018 on tomato pomace (TP) residues obtained during 2017 processing tomato season kindly provided by SSICA, Angri Italy.
Manure of non-milking Mediterranean water buffalo was collected in a commercial buffalo farm located in Villa Literno, South West Italy (Carillo et al., 2012a). Bacteria from buffalo dung (BB) were grown and selected on a specific Clostridial Nutrient Medium (Sigma-Aldrich). In addition, commercial strains of lactic acid producing bacteria (LB) *Lactobacillus rhamnosus* GG (ATCC 53103) (Valio Ltd, Finland) were cultivated and enriched on MRS selective broth (SIGMA-ALDRICH) favouring the growth of Lactobacilli. Then, the two types of bacteria consortia were used as inoculum alone or in combination for the anaerobic fermentation of TP as substrate for the production of fermentable sugars and their conversion to LA.
Two types of substrates were used in the experimental tests: TP as it was (fresh) and TP undergoing a pre-heating treatment at 70 °C lasting 72 h (pre-processed). Fresh and pre-processed TP underwent TS, VS, soluble sugars and cellulose analyses. Since sugars concentration in the pre-processed TP was higher than that of fresh TP, the former was chosen as a substrate for the anaerobic digestion process. Pre-processed TP was gently mixed with tap water, with a weight ratio of 1/30, and the mixture gently stirred and acidified, with a solution of HCl to obtain the desired pH values. The pH value of the original water/pomace solution was around 7.5 and it was corrected to 7.0, 6.0 and 5.0. Then, the water/pomace solution was homogenized to simulate a stressing mechanical treatment and used as it, or inoculated with LB, BB or LB + BB. The anaerobic fermentation process was performed in batch mode in tubes filled with 30 mL of pomace mixture, flushed with oxygen free nitrogen to assure anaerobiosis and finally placed in an incubator at controlled temperature of 37 °C to start the fermentation process and continuous mixing was applied. The experiments were performed in triplicate.

2.1 Total and Volatile solids

The total solid (TS) content was determined upon oven dehydration at 105 °C (until steady weight). For the evaluation of volatile solids (VS), TS were placed in ceramic crucibles and ignited in a muffle furnace at 550°C for 60 minutes each cycle. Three heating cycles were performed to attain a steady weight measurement. Then the crucibles, after taken out of the muffle furnace and partially cooled in air, kept in desiccators for few minutes were weighed. The average values were determined using five different samples. The calculation of the TS (or dry matter) is specified by the European Standard (UNI EN 14346, 2007) as reported in Guarino et al. (2016). This method applies to solid samples with dry weight greater than 1 % and samples which become solid during the drying process. The calculation of the VS of samples is specified by the European Standard (UNI EN 15169, 2007) (Guarino et al. 2016). This procedure is applicable to all kinds of waste, sludge and sediments and it is often used to estimate the content of volatile organic matter.

2.2 HPLC-Dionex Measurements

LA was assayed directly after fermentation from the centrifuged fermented broth by ion-exchange chromatography using a DX500 apparatus (Dionex, Olten, Switzerland) equipped with an IONPAC-CTC cation trap column (Dionex), an IONPAC-CG12A guard column (Dionex) and an analytical IONPAC-CS12A 4-mm column (Dionex), fitted with a CSRS 4-mm suppressor (Dionex), with detection by a CD20 conductivity detector (Dionex), according to the manufacturer's instructions (Carillo et al. 2011).

2.3 Sugars Measurement

The amount of ethanol-soluble and alkali-soluble sugars and the glucose released from tomato pomace during the cellulose digestion by from Trichoderma reesei ATCC cellulose (Sigma-Aldrich) were determined in a microplate assay followed with a spectrophotometer Xenius (Safas, Montecarlo), using an enzyme-coupled

colorimetric reaction, highly specific for glucose according to (Carillo et al. 2012b; Woodrow et al. 2017). Sugars were expressed as percentage of TS.

2.4 DGGE Analysis

The analysis of the microbial community was carried out using denaturing gradient gel electrophoresis (DGGE) to generate fingerprints of 16S rRNA genes (Carillo et al., 2012a). Nucleotide sequences (Operational Taxonomic Units, OTU) were compared to the GenBank-NCBI nucleotide database using the BLAST network service as described in Carillo et al. (2014).

3. Discussion of Results

Results in Table 1 show the main physical and chemical composition of fresh and 70 °C for 72 h pre-processed TP. All the measured components reported in Tables and Figures are expressed as percentage of TS (% TS), whereas TS and VS are calculated with reference to fresh substrate mass. Volatile solids are shown for evaluating the presence of carbon and other ignitable matter, which in principle can be converted into valuable products. Glucose, fructose and alkali-soluble sugars are present in very low amount (% TS) in the fresh substrate. When pre-processed substrate is taken into account, soluble sugars and cellulose show larger concentrations due to cell walls weakening probably because of the disruption of hydrogen bonding between plant cell wall polymers (McQueen-Mason and Cosgrove, 1994). Fructose is the largest sugar present in TP. Cellulose concentration was higher in the pre-processed TP and equal to 2.30 % TS, and about 30 % higher than fresh TP.

Table 1: Chemical and physical characterization of fresh and 70 °C for 72 h pre-processed TP. The numbers are the Mean values, while those in parenthesis represent the corresponding Standard Deviation

Tomato Pomace	TS (%)	VS (%)	Glucose (% TS)	Fructose (% TS)	Alkali-soluble sugars (% TS)	Cellulose from soluble fraction (% TS)
Fresh	65.8 (4.0)	58.3 (1.8)	0.22 (0.09)	0.40 (0.07)	0.14 (0.05)	1.81 (0.33)
Pre-processed			0.28 (0.08)	0.86 (0.25)	0.44 (0.26)	2.30 (0.21)

In Table 2 the same sugars were measured after 16 h of anaerobic digestion process (using an initial value of pH 7.0). It is clear that they are reduced to very low quantities, which can be considered nearly negligible. On the other side, it is very interesting to observe that LA concentration shows moderately high values, except when TP was not inoculated with bacteria. The largest LA concentration is found when both LB and BB inocula are provided. Anyway, the large standard deviation observed with the BB inoculum does not allow to state at the moment which is the best solution for LA production. It needs to be stated, anyway, that these results are preliminary and more tests are required to identify the best processing conditions for LA production. The initial choice of measuring LA and soluble sugars after 16 h was based on the observation that the exponential microbial growth phase lasted 16 h, followed then by the stationary phase.

Table 2: Soluble sugars and acid lactic content of pre-processed TP anaerobic digestion, initial pH 7.0 with and without bacteria inoculum. The numbers are the Mean values, while those in parenthesis represent the corresponding Standard Deviation

Tomato Pomace	Glucose (% TS)	Fructose (% TS)	Alkali-soluble sugars (% TS)	Lactic acid (% TS)
No inoculum	0.01 (0.00)	0.02 (0.00)	0.04 (0.01)	0.19 (0.03)
LB inoculum	0.03 (0.02)	0.02 (0.00)	0.06 (0.01)	1.36 (0.13)
BB inoculum	0.04 (0.01)	0.04 (0.00)	0.10 (0.02)	1.43 (0.54)
LB+BB inoculum	0.04 (0.01)	0.03 (0.02)	0.12 (0.01)	1.69 (0.10)

Figure 1 shows the LA production after 16 h TP anaerobic digestion run with initial pH equal to 5.0, 6.0 or 7.0, and temperature equal to 37 °C. The best LA production recorded after 16 h digestion of 2.16 % TS is obtained by tomato pomace inoculated with LB and BB, while non-inoculated and LB inoculated tomato pomace are less productive, independently of initial pH value. LA production rate is shown to be faster in LB + BB treatment (Figure 2), but after 5 h of digestion, when an LA value of about 4.3 % TS is obtained, LA concentration starts decreasing. On the contrary, LB and BB inocula are initially slower, attaining the

maximum LA value after 10 h, with concentration values equal to 2.78 and 3.31 % TS, respectively, and then start decreasing, even faster than LB + BB treatment does (Figure 2).
The final pH values, measured at the end of the 16 h digestion process (Figure 1), show that there is a gradual decrease of pH independently of the initial pH value, due to the production of LA and other organic acids, which acidify the media. The lowest pH of 3.9 is attained considering the media with an initial value pH 5.0 and is independent of the bacteria inoculum.

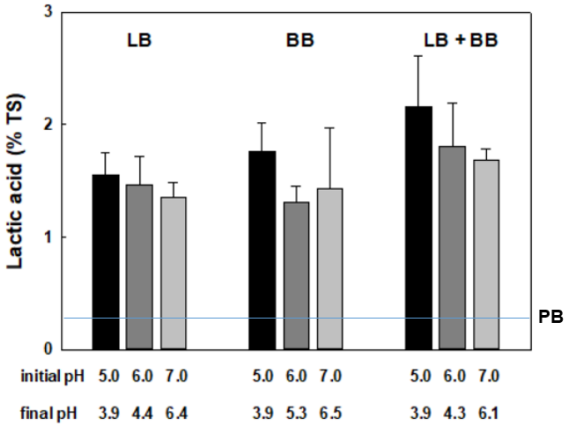

Figure 1: Lactic Acid production (% TS) for different pH tested values and employed bacteria communities.

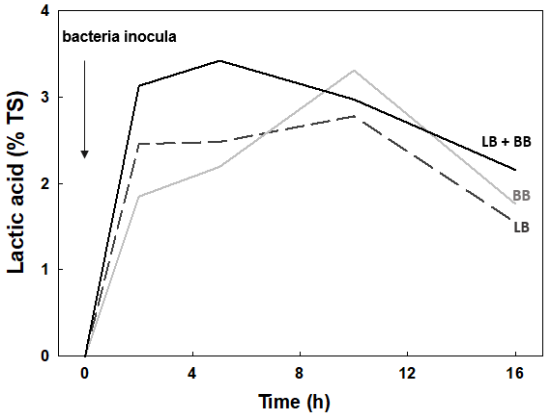

Figure 2: Lactic acid time evolution (% TS) for different employed bacterial communities in the fermentation process.

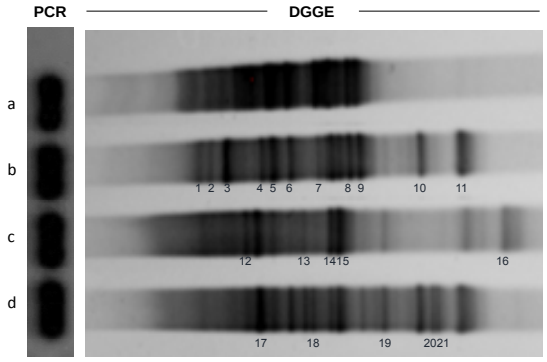

Figure 3: PCR (Polymerase Chain Reaction) and DGGE (Denaturing Gradient Gel Electrophoresis) profiles of Eubacteria 16S-rRNA gene sequences from a) non inoculated, b) lactic acid bacteria (LB) inoculated, c) buffalo bacteria (BB) inoculated, d) LB + BB inoculated fermented tomato pomace.

In Figure 3 the PCR and DGGE study of tomato pomace digestion bacterial microflora is shown. Twenty-one different fragments (gel bands) of similar size were eluted and sequenced after reamplification from DGGE gel. Search by GenBank database using the BLAST network service, show that 18 of 21 had high identity (86–100 %) to V3 16S-rRNA gene sequences belonging to previously characterized microbes (Table 3). Seventeen out of 21 are bacteria residing in waste digesters, anaerobic sediments, tomato pomace silage or faecal samples. Only the sequences 1, 2 and 16 show low identity to other 16S-rRNA gene sequences present in the nucleotide databases, resulting still unknown. Fifteen out of 18 eubacterial 16S-rRNA gene sequences can be assigned to the phylum Firmicutes and three to the phylum Bacteroidetes.

Table 3. Uncultured bacterium 16S ribosomal RNA partial sequences isolated by DGGE from TP batch fermentation with and without bacteria addition at pH 7.0 and 37 °C.

OUT	**Phylogenetically most closely related Eubacteria**			
	Description – accession n°	Sm (%)	Phylum	Source
1, 2	Uncultured bacterium HQ327483.1	80-85	-	Germany: MWSW and algae bioreactor
3, 4, 5, 6	*Lactobacillus plantarum* KF312679.1	89-94	Firmicutes	China: tomato pomace silage
7	*Bacillus siralis* NR_028709.1	89	Firmicutes	Sweden: silage
8	*Ethanoligenens harbinense* YUAN-3 *Clostridium sp.* - CP002400	91	Firmicutes	China: mesophilic digester
9	*Acetanaerobacterium elongatum strain Z7* - NR_042930	86-93	Firmicutes	China: paper mill waste water
10, 11	*Lactobacillus rhamnosus* GG (ATCC 53103)	100	Firmicutes	Finland: purchased by Valio Ltd
12, 17	Uncultured Clostridia EU887985.1	93-95	Firmicutes	Finland: leach bed reactor
13, 18, 19, 20, 21	Uncultured bacterium EU551121.1	91-96	Bacteroidetes	Finland: anaerobic co-digestion of crops and cow manure
14, 15	*Clostridium populeti* NR_026103.1	96-98	Firmicutes	USA: cellulose culture
16	Uncultured bacterium - JF573812.1	83	-	USA: anaerobic fermentation of rumen fluid and sediments

Eubacterial bands 3, 4, 5, 6 are particular evident in figure 3 a and b, and show a similarity of 89-94% to *Lactobacillus plantarum*, which promotes LA fermentation and improves tomato pomace silage quality (Wu et al. 2014). However, when used on maize silage, Đorđević et al. (2017) found that it increased acetic acid but not lactic acid concentration. Accordingly, LA concentration in non-inoculated tomato pomace was on average 0.19 % TS only. Moreover, when tomato pomace was inoculated only with BB, *L. plantarum* bands decreased, suggesting that the BB rapid growth overtakes these bacteria. Sequence 7, present in figure 3 a and b, show a 89 % similarity to *Bacillus siralis*, an anaerobic bacterium which uses soluble sugars and is able to hydrolyse casein but not starch or cellulose (Petterson et al. 2000). Sequence 14 and 15 have a 96-98 % similarity to *Clostridium populeti*, which can produce hydrogen and lactic acid from glucose and cellulose (Sleat and Mah, 1985). Sequences 10 and 11 have a 100 % identity to *Lactobacillus rhamnosus* GG (ATCC 53103), and correspond to the commercial bacterial strand used as LB inoculum. *L. rhamnosus* is widely studied due to its use as probiotics, ability to survive in acid environments and its lack of pathogenicity. It is able to use both cellulose and hemicellulose-derived sugars for lactic acid production and not only glucose as homofermentative strains of lactic acid bacteria like *Lactobacillus acidophilus* (Cui et al. 2011). It is evident that when mixed co-cultures of LB and BB bacteria are present, these microorganisms may increase the conversion efficiency of substrates to LA (Nancib et al. 2009) (Figures 1 and 2).

4. Conclusions

Anaerobic fermentation of tomato pomace was performed using different pH values of the substrate, with or without pre-heating the substrate. Results show that without using appropriate inocula of bacteria consortia negligible amount of LA are produced, even though fermentable sugars are initially present in the fresh TP. Pre-heating of TP increases the amount of fermentable sugars, included glucose released from hydrolyzable cellulose. Using LB and/or BB inocula, higher LA production is obtained with the consortium of the two inocula giving the best results. Notwithstanding the exponential microbial growth phase lasted for 16 h, the LA amount obtained after 16 h anaerobic digestion is not the largest one. Instead, for the LB+BB consortia inoculum, the best production was obtained after only 5 h. This suggests that shorter measurements during the fermentation process are required to check the most effective LA production. This will allow to design efficient biological processes aimed at achieving the highest LA recovery yields, potentially coupled with biogas production.

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