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Unlocking the potential of food industry by-products: Sustainable volatile fatty acids production via mixed culture acidogenic fermentation of reground pasta

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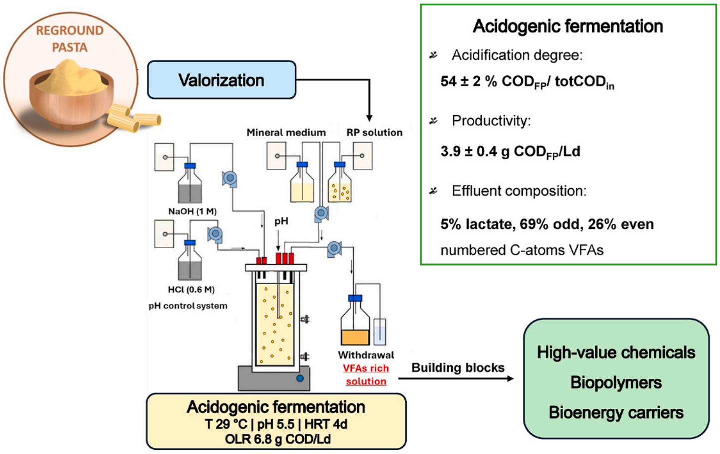
HIGHLIGHTS

- Reground Pasta was valorised through fermentation into volatile fatty acids (VFA).
- Chemical Oxygen Demand (COD) balance indicates a high level of conversion efficiency.
- Water stirring pre-treatment batch tests improved hydrolysis by 20.2 % COD/totCOD$_{in}$.
- 54 % acidification degree and 18.7 gCOD$_{VFA}$/L were obtained in the acidogenic effluent.
- 91 % of totCOD entering the acidogenic reactor was accounted as VFA, biomass and H_2.

GRAPHICAL ABSTRACT



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ABSTRACT

The agroindustry generates substantial by-products each year, creating disposal challenges. In this view, this study focused on the valorisation of reground pasta (RP), a farinaceous by-product, towards the production of volatile fatty acids (VFAs), direct precursors to produce higher commercial value compounds (polymers, bioenergy or food conservatives), through mixed microbial culture (MMCs) fermentation. The RP solution contains

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Volatile fatty acids (VFAs)
Sequencing batch reactor (SBR)

high organic carbon (99 % volatile solids), which indicates strong potential for acidogenic conversion into fermented products. First, by using pre-treatments (water stirring, pH change, sonication and boiling) in batch mode, its hydrolysis degree was evaluated. After water stirring was chosen as pre-treatment, the RP solution thus obtained was fed to a lab-scale sequencing batch reactor (SBR). A conversion yield into fermentation products (FP) of 54 ± 2 % in terms of Chemical Oxygen Demand ($COD_{FP}/totCOD_{in}$) was obtained with a concentration of 18.1 ± 1.0 g COD_{VFAs}/L composed by 69 % odd (6.5; 3.8; 2.7 gCOD/L of acetic, iso-butyric and butyric acids, respectively) and 26 % even (4.0 and 1.0 gCOD/L of propionic and valeric acids, respectively) numbered C-atoms VFAs. Microbial community analysis revealed the dominance of *Prevotella* and *Clostridium* genera, which contributed to the production of propionic and acetic-butyric acids, respectively. These results, combined with the novelty of a pasta by-product valorisation through MMCs fermentation, unlock new pathways for sustainable waste valorisation and the production of high-value bioproducts.

1. Introduction

Nowadays, to ecologically satisfy the global market for fermentation products, it is mandatory to identify renewable resources to produce sustainable chemicals (Agnihotri et al., 2022). Industrial fermentation (IF) represents a relevant biotechnology platform where organic materials can be converted into valuable chemicals like organic acids, amino acids, alcohols, solvents, and enzymes (Lee et al., 2011). Among these, volatile fatty acids (VFAs) are highlighted for their significant industrial applications as building blocks for producing chemicals, bioenergy and biopolymers (Marchetti et al., 2025; Marzulli et al., 2023; Zeppilli et al., 2017). VFAs can be produced through acidogenic fermentation (AF) involving hydrolysis and acidogenesis stages (Laiq Ur Rehman et al., 2019), and various strategies: adjusting temperature, pH, and organic loading rate (OLR) can enhance their production (Marchetti et al., 2024; Soomro et al., 2020).

AF of heterogeneous and undefined food wastes (such as fruit, vegetable, or dairy by-products) has received recently considerable attention and the topic has been covered in comprehensive review papers (Gottardo et al., 2022; Strazzera et al., 2018). Indeed, the use of by-products and food-waste as substrates is widely recognized as a sustainable strategy, as it supports the principles of circular bioeconomy by promoting resource recovery, reducing waste generation, and minimizing environmental impacts (Leong et al., 2021). In contrast, AF potential of by-products from the manufacturing of edible pasta remains largely underexplored. Considering that the global farinaceous products market reached a size of 250.8 bilion USD in 2023, with 376 kilotons of pasta-waste produced every year (Sîrbu, 2023), its valorisation should become a route of ever-increasing relevance. Hence, in the present study the reground pasta (RP), a by-product from farinaceous food industry, was used as substrate and its fermentability was investigated.

Moreover, previous research regarding this by-product/waste has mainly relied on pure cultures to obtain specific acids, requiring strict operational conditions and high costs, as in Zhang et al. (2013) where through *Actinobacillus succinogenes*, bakery waste was evaluated for its succinic-acidogenic potential, or also in López-Gómez et al. (2022), where six *B. coagulans* were used to evaluate the fermentation of pasta wastes in the lactic acid production. In this view, the purpose of the present work was the valorisation of the RP solution through an acidogenic fermentation in a lab-scale reactor, that explores the novelty of the advantageous use of MMCs instead of single strains, thus enabling a more flexible and robust process, less dependent on strict operational control, and better suited for treating variable and complex real waste matrices.

Complementarily, several studies also emphasize the importance of pre-treating carbohydrate-rich substrates to optimize the hydrolysis, which is the main kinetic limitation step (Taherzadeh and Karimi, 2008). Hydrolysis, breaking down complex components into simpler sugars, increases soluble COD in the solution easily utilized by fermenting bacteria, enhancing the overall process yield. For this reason, the present study, also looked to the possibility, through different batch pre-treatments, to break the complex constituents as carbohydrates like starch (comprising 70 % wt/wt) or fibers (comprising 2 % wt/wt) naturally present in the RP by-product (Pasqualone et al., 2017). In conclusion, by integrating MMCs in the acidogenic fermentation, the proposed approach supports the circular valorisation of food waste streams, contributing to the development of a resilient biorefinery system, paving the way for sustainable high-value bio-based chemicals, biopolymers, and bioenergy production routes.

2. Material and methods

2.1. Pre-treatment of RP

A preliminary study conducted on the RP hydrolysis degree was carried out by water stirring pre-treatment, at room temperature (25 °C), three solutions: 0.8 g RP/L (solution A), 1.8 g RP/L (solution B) and 6.14 g RP/L (solution C) and following the hydrolysis every hour up to 4 h. Afterwards, the solutions were placed in the fridge at 4 °C and sampled for an additional 3 days to monitor the release of nutrients and soluble COD from the RP into the solution. Additional batch tests were made starting with solution C for each pre-treatment: chemical through pH adjustment, physical through sonication, and thermal by boiling, where the control test consisted of the solution's initial values. The chemical pre-treatment was conducted in duplicate for each pH value, stirring the bottles containing RP with addition of NaOH (3M) and HCl (3M), while the control test was performed at pH 7. The solution was changed to acidic and alkaline values of 2, 4, 6, 8 and 10, evaluating after 1 h the effect on the solution hydrolysis. Following, tests were rerun choosing pH values of 2 and 10 (tests in quadruplicate), where sampling was conducted after 10 min of incubation at the target pH and afterwards, pH was readjusted to 7 and re-sampled after 10 min (pH2 → 7 and pH10 → 7). Physical pre-treatment tests consisting in sonication, conducted in duplicate, were applied to the RP solution for 30 min in a water bath at room temperature (25 °C) using the low frequency (20 kHz) sonication unit developed by Ultrawaves GmbH (Hamburg, Germany) (Li et al., 2018). Thermal pre-treatment was carried out in duplicate by heating the RP solution under stirring reaching 100 °C and sampled after 5 and 15 min upon reaching the boiling point.

2.2. Operation of the AF reactor

The AF process was carried out in an SBR, with a total working volume of 1.1 L, inoculated with an anaerobic sludge and operated with a Hydraulic Retention Time (HRT) of 4 days. A schematic reactor diagram is reported in Fig. S1. Feeding took place every hour (11.46 mL in 1 min, 24 times per day, for a total of 275 mL/day) via two pumps, one pumping 75 % (v/v) of the RP solution and the other pumping the remaining 25 % (v/v) of mineral medium which contains the necessary components for microbial growth, reported elsewhere (Fradinho et al., 2013). The AF reactor temperature was maintained at 29 ± 1 °C through a thermostatic bath, and the pH was controlled at 5.50 ± 0.01, using NaOH (1 M) or HCl (0.66 M), to inhibit methanogenic activity. The OLR was set to 10 g CODtot/Ld, using a concentrated solution of 53 g COD/L obtained by adding 47g of RP in 1 L of distilled water (1.14 g COD/g RP conversion factor was used according to Marchetti et al., 2023). The RP feeding solution, with a pH equal to 6, was periodically replaced, kept at 4 °C under mixing conditions and sampled every 2 days for acids, solids, sugars, nutrients and COD content characterization along the time. In particular, the applied OLR trend was determined by measuring the COD of the RP solution at each new bottle replacement. The concentrations of ammonia (N), phosphorus (P) and sulphate (S) components, in the mineral solution, were set considering the applied OLR and using the general formula for anaerobic biomass $CH_{1.8}O_{0.5}N_{0.2}S_{0.002}P_{0.02}$ (Stephanopoulos et al., 1998). Using this formula, the nitrogen, phosphorus and sulphur volumetric rates, provided by the mineral medium, were equal to 62.1 mg N/Ld, 13.8 mg P/Ld and 1.42 mg S/Ld, respectively. Lastly the sugar volumetric rate, from the RP solution fed to the reactor, was 1.18 ± 0.3 g $Sugar_{tot}$/Ld.

2.3. Analytical methods and microbial community assessment

Total/volatile solids and suspended solids (TS and VS, TSS and VSS) concentrations were measured according to Standard Methods and converted in COD unit by using the conversion factor of 1.42 g COD/g obtained from the complete oxidation of heterotrophic biomass (APHA, 1995). Ammonia and phosphate concentrations were measured by colorimetry using a segmented flow analyser (Skalar, The Netherlands) (Arias et al., 2018). Ammonia determination was based on the modified Berthelot reaction (Krom, 1980); phosphate through the formation of the antimony-phosphomolybdate complex measured at 880 nm. The total and soluble chemical oxygen demand (totCOD and solCOD) were measured by digestion of the total and the filtered (0.2 μm) sample, with dichromate at 150 °C according to Standard Methods (APHA, 1995). Monosaccharides (fructose and glucose), sucrose, lactate, acetic, propionic, iso-butyric, butyric, iso-valeric, valeric and caproic acids were determined by high performance liquid chromatography (HPLC), detailed information is provided in (Marchetti et al., 2024). All samples were analysed after centrifugation at 13.000 rpm for 3 min and filtration through a 0.2 μm membrane. The sum of the fermentation products (lactate, acetic, propionic, isobutyric, butyric, isovaleric, valeric and caproic acids) and the sugars were expressed as COD by using the corresponding conversion factor for each acid, reported in Supplementary in Table S1. Total sugars were determined using the Dubois method (DuBois et al., 1956) measured by spectrophotometer at 490 nm. The reactor gas outlet flow was determined by a flowmeter (μFlow unit, BPC Instruments, Sweden) and the gas composition (CH_4, CO_2, O_2, N_2 and H_2) was determined using a gas chromatograph (Agilent Technologies, 7890B, USA) (Marchetti et al., 2024). The gas was sampled from a gas sampling point using an airtight syringe and immediately injected in the GC. Microbial communities from the AF reactor were identified through metagenomic analysis, extracting DNA from all organisms. In detail, samples were analysed using gene amplicon sequencing targeting bacterial 16S rRNA gene variable region 1–3 (bV13-A) in combination with taxonomic classification against the MiDAS 2.1.3 database, prepared by a custom protocol according to Caporaso et al. (2012) using the specific primers [27F] AGAGTTTGATCCTGGCTCAG and [534R] ATTACCGCGGCTGCTGG (Group, 2012). The resulting Sequencing libraries were purified using the standard protocol for CleanNGS SPRI beads (CleanNA, NL) with a bead to sample ratio of 4:5. DNA was eluted in 25 μL of nuclease free water (Qiagen, Germany). DNA concentration was measured using Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, USA). Gel electrophoresis using Tapestation 2200 and D1000/High sensitivity D1000 screentapes (Agilent, USA) was used to validate product size and purity of a subset of sequencing libraries.

2.4. Calculations

The solubilization degree of the RP solution along the system operation (%, solCOD/$totCOD_{in}$) was calculated as reported in equation (a). Also, considering that the fermentation products (on COD basis) entering the reactor was zero, the degree of fermentation (DF) was defined as reported in equation (b). Therefore, the productivity of fermentation products (FP) in respect to the RP solution flow incoming the reactor, was calculated by dividing the products concentration by the HRT of 4 days. The calculation was made for each sampling day by comparing the acids concentration obtained from HPLC analysis to the total COD measured on the same day. The same calculation was made referring to the soluble COD. The equation used is given below:

$$Solubilization\ (\%) = \frac{\Sigma solCODout - \Sigma solCODin}{\Sigma totCODin} = \frac{\overline{solCOD}}{\overline{totCODin}} \tag{a}$$

$$DF\ on\ total\ or\ soluble\ COD\ (\%) = \frac{\Sigma CODFP}{\Sigma tot\ or\ sol\ CODin} = \frac{\overline{CODFPout}}{\overline{tot\ or\ sol\ CODin}} \tag{b}$$

All other calculations regarding the conversion of the total COD_{in}, in terms of acids (degree of acidification - DA), sugars residue in the effluent (quantified by Dubois method), biomass and hydrogen are reported in Supplementary: equations s1, s2, s3 and s4, respectively.

3. Results and discussion

3.1. Pre-treatment batch tests of RP

Before the reactor start-up, it was evaluated the RP solution hydrolysis, before its storage in the fridge. First, a preliminary study on the hydrolysis of RP in two solutions (solution A 0.8 g RP/L – solution B 1.8 g RP/L) was performed using a water stirring pre-treatment. The obtained results showed an increase in ammonia, ranging from 0.05 mg N/L to a maximum of 0.09 mg N/L, and from 0.11 ± 0.01 mg N/L to 0.21 ± 0.01 mg N/L, for solution A and B, respectively after 3 days. This can be explained by a release of ammonia into solution, and subsequent deamination of amino acids, through the breakdown of proteins (considering nitrogen in protein averaged 16 %), which can be around 15 g/100 g in flour products (Pasqualone et al., 2017). The stability of RP constituents following hydrolysis was also assessed. Soluble COD reached stable values by the third hour of the test, keeping this solubilization degree fairly unchanged after 72 h, with a final value around 5.6 %, 10 % and 20.2 % at 4 °C, for solution A, B and C (6.14 g RP/L ~ 7 g CODtot/L), respectively (Fig. S2). Afterwards, to evaluate the possibility of increasing the hydrolysis of RP solution, starting to solution C, three additional pre-treatments (chemical, thermal and physical) were performed, namely, change of the pH, temperature and sonication. Results for soluble COD indicated a similarity between the control test and the tests conducted at pH 4, 6, and 8, while significant increases in soluble COD were observed at pH 2 and pH 10 (Fig. S3). For this reason, a second set of tests were carried out only for pH 2 and 10. Table 1 shows the results of the pre-treatment tests performed on the RP solution, in terms of soluble COD, nutrients and solids content.

By comparing the initial soluble COD before (control equal to 2.4 g COD/L) and after pre-treatment, a slight increment for pH 2 and pH 10 was observed (3.1 and 2.9 g COD/L, respectively), while with the sonication, the soluble COD remained almost unchanged (2.6 g COD/L). On the other hand, for boiled RP solution there was a major increment in soluble COD compared with the soluble COD of the control test, reaching a solubilization degree of 27.7 % (solCOD/$totCOD_{in}$) after 15 min from reaching boiling point. This result indicates thermal treatment increased hydrolysis degree of more complex RP organic matter. Focusing on the nutrient content, there was no real change in terms of phosphate content, remaining at around 3.30 mg P/L, while in case of the boiled solution and sonication treatments the ammoniacal nitrogen content changed from 0.40 ± 0.01 mg N/L in the control test to 0.90 ± 0.05 and 0.80 ± 0.01 mg N/L, respectively. Hence, the natural consequence of the effect of hydrolysis triggered by water (seen in solutions A, B and C) was sped up here. Accordingly, the most promising pre-treatment to maximize RP hydrolysis was the thermal one. Further tests could be carried

Table 1
Results obtained from pre-treatments of RP solution with a concentration of 7.0 g totCOD/L of RP (n = 4 for pH 2 and pH 10; n = 2 for boiling and sonication tests).

	Pre-treatments							
Parameters	**Control**	**Water$_{stir}$**[a]	**pH 2**	**pH 2 → 7**	**pH 10**	**pH 10 → 7**	**Heat**	**Sonication**
solCOD(gCOD/L)	2.4 ± 0.05	3.8 ± 0.05	3.1 ± 0.1	2.9 ± 0.03	2.9 ± 0.02	3.1 ± 0.03	4.2 ± 0.01	2.6 ± 0.05
NH_4^+ (mgN/L)	0.4 ± 0.01	0.7 ± 0.01	0.4 ± 0.01	–	0.4 ± 0.02	–	0.9 ± 0.05	0.8 ± 0.01
PO_4^{3-} (mgP/L)	3.5 ± 0.11	3.5 ± 0.05	3.2 ± 0.06	–	3.4 ± 0.09	–	3.3 ± 0.02	3.5 ± 0.24
VSS/TSS	0.9 ± 2 E–0.5	0.9 ± 0.01	0.9 ± 5 E–0.5	0.9 ± 0.01	0.9 ± 8 E–06	0.9 ± 4 E–0.5	0.9 ± 0.01	0.9 ± 0.01

[a] After 4 h.

out, investigating the combination of the different methods, which can lead to a greater increase in the degree of solubility. As an example, in Vavouraki et al. (2014), chemical pre-treatment of kitchen wastes (KW), with HCl and KOH solutions, was conducted in combination with enzymatic hydrolysis. Also, in Hafid et al. (2015), where HCl and H_2SO_4 mild acid solutions and enzymatic hydrolysis (glucoamylase) pre-treatments were tested on KW, a solubilization of 40 % was obtained by the combination method of 1.5 % HCl and glucoamylase. In Kumar and Mohan (2018), where vegetable wastes were subjected to physical (PSC), chemical (CHE), and their combination pre-treatments, the individual pre-treatments resulted in lower degree of solubilization (PSC: 37.3 %, CHE (H_2SO_4/HCl): 30/27 %, NaOH/(CaOH) 2: 21/20 %), while the combination of PSC and CHE with acid-catalysed conditions accelerated the sugars solubilization (58 %). However, in the present study, by simply stirring the RP solution with water for up to 4 h at room temperature, a 20.2 % solubilization degree was obtained. This slight difference, considering also the energy demand and operational complexity associated with boiling large volumes of solution, determined that utilizing the RP solution with water stirring pre-treatment was the preferred approach. Moreover, this permitted the evaluation of the inherent fermentability of RP solution, thus providing a valuable benchmark, establishing a realistic baseline against which future studies, involving pre-treatment strategies can be compared.

3.2. AF reactor operation with RP solution as a feedstock

3.2.1. Characterisation of the RP solution feedstock along the AF reactor operation

To evaluate its acidogenic potential, the RP solution fed to the reactor was monitored in terms of sugars, COD and solids content in the reactor inlet, throughout the days (Table 2). This characterisation was essential to define the substrate's fermentability potential and to tailor the feeding strategy and operational settings ensuring optimal acidogenic fermentation conditions.

The percentage of VSS in respect to TSS, which represents the organic fraction, was higher than 99 %, highlighting the high organic transformation potential of this substrate. The analysis of the RP solution in HPLC showed that the largest constituent, in terms of soluble sugar, was sucrose (5.2 ± 0.1 g solCOD/L), followed by fructose (0.13 ± 0.002 g solCOD/L) and glucose (0.03 ± 0.0005 g solCOD/L), corresponding to a soluble sugar concentration of 5.4 ± 0.1 g_{Sugar} solCOD/L fed to the reactor (15.8 ± 0.2 % of the total inlet COD). These sugars are the components readily accessible as for bacteria. The remaining sugar constituents, defined with Dubois, was likely more complex sugars, such as polysaccharides (starch), which in the fermentative process need to be hydrolysed by hydrolytic bacteria. Overall, the soluble COD concentration of the RP solution fed to the reactor accounted for 37 % of the total COD. By characterising the inlet RP solution during the working days, a value of 34.0 ± 1.0 g totCOD/L was obtained, corresponding to an experimental OLR of 6.8 ± 0.2 g totCOD/Ld, lower than the set value (10 g totCOD/Ld). Despite the RP by-product used during the batch tests in Marchetti et al., (2023) and in the present study, being supplied by the same company, this difference could be attributed to the complexity and variability of real waste-derived substrates, not compromising the authenticity of the results obtained.

Table 2
Characterization of RP solution entering the AF reactor in terms of sugars and Chemical Oxygen demand (COD). (n = 6 along the working days of AF reactor).

Parametric characteristics of the RP solution	
COD_{tot} (gCOD/L)	34.0 ± 1.0
COD_{sol} (gCOD/L)	12.4 ± 0.5
$Sugar_{tot}$ (gCOD/L)	6.3 ± 1.6
$Sugar_{sol}$ (gCOD/L)	5.4 ± 0.1
COD_{sol}/COD_{tot} (%, COD/COD)	37.0 ± 0.6
$Sugar_{sol}/Sugar_{tot}$ (%, COD/COD)	85.7 ± 1.0
$Sugar_{tot}/COD_{tot}$ (%, COD/COD)	18.5 ± 0.9
$Sugar_{soluble}/COD_{tot}$ (%, COD/COD)	15.8 ± 0.2

3.2.2. COD solubilization degree

During the reactor operation, the soluble COD leaving the reactor increased over time, stabilizing by day 55th. As shown in Fig. 1, the obtained solubilization degree during the 96 working days ranged from an initial value of 12.8 ± 4.5 % to a maximum value of 61.3 ± 1.8 % (g solCOD/g totCOD) around the 55th day, with the residual COD not converted into fermentation products being used for biomass growth or biogas production. This progressive increase in solubilization degree reflected the adaptation of the microbial community to the process. To know the concentration of total and soluble COD entering the system, samples of the RP solution fed to the reactor were analysed (violet and yellow trends, respectively). The results indicate that the outgoing soluble COD follows a similar pattern to the incoming total COD. This suggests that during the acidogenic fermentation process, RP was easily hydrolysed. Besides, in Fig. S4, the ratio between the obtained VFA (on COD basis) during the AF reactor working days on the total and soluble COD in the outlet effluent, was reported. This information is extremely useful with a view of future using the fermented solution, as it allows to know the amount of acids present in the COD outlet, and thus the percentage of substrate readily useable as chemical building blocks. In detail, an average value equal to 45.1 ± 1.5 % and to 58.6 ± 1.8 % (COD/COD), in terms of VFAs concentration in the total and soluble

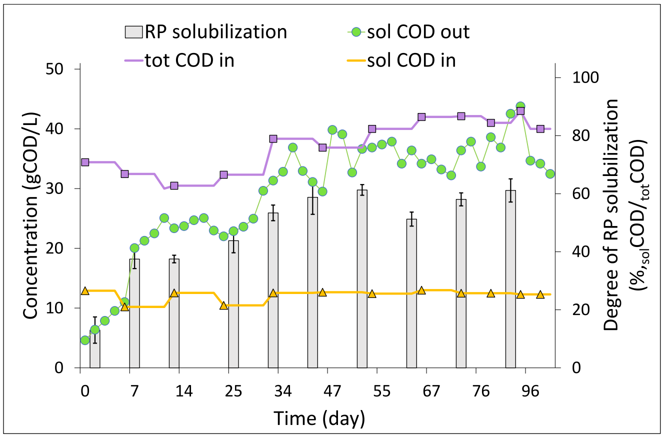

Fig. 1. Average of solubilization degree and trends of soluble and total COD of RP solution incoming and outgoing the acidogenic reactor along the working days of reactor.

outlet COD was obtained, respectively. While 13.5 ± 1.2 % was the non-soluble COD component present in the outlet not converted into acids, hardly useable.

3.2.3. *Characterization of the fermented effluent*

During the 96 working days, the effluent leaving the AF reactor was analysed in terms of carboxylic acids, sugars, nutrients and solids content. In Fig. 2A, the average of total and soluble sugar volumetric rate, incoming and outgoing the reactor are reported, which corresponded to a daily consumption of 72.2 ± 1.3 % and 91.9 ± 1.2 % (COD/COD), respectively, showing a good consumption of sugars. The amount of non-soluble sugars consumed by the microbial culture in the reactor was around 48 % of the 0.40 g_{Sugar}/Ld, highlighting the enzymatic hydrolytic activity of the microbial community, likely involving extracellular amylases and glycosidases, enabling the breakdown of complex carbohydrates present in RP into fermentable monomers before acidogenesis. A concentration equal to 0.26 ± 0.02 g COD/L and 1.2 ± 0.07 g COD/L of soluble and total sugars in the fermented effluent was obtained during the reactor operation. This means that only ~3 % ($COD_{Sugartot}$/totCOD) was not converted into acid products during the fermentation process.

For the nutrients content, the average volumetric rates incoming from the mineral medium and outgoing the reactor, are reported in Fig. 2B. Considering the outgoing nutrients equal to 2.17 mg P/Ld and 7.15 mg N/Ld, a consumption of 11.6 ± 0.1 mg P/Ld (84.2 ± 0.9 %, wt/wt) and of 55.0 ± 0.6 mg N/Ld (88.5 ± 1.0 %, wt/wt) for phosphate and ammonium was obtained, meaning that nutrients were not limiting bacterial growth in the reactor.

Regarding the solids content, values of 8.2 ± 0.2 g/L and 7.6 ± 0.2 g/L for total and volatile suspended solids were obtained (Fig. S5), with a VSS/TSS ratio accounting for around 93 % (wt/wt) and the mineral content ratio for 7 %. VS were ≈64 % (wt/wt) of the TS, with a higher mineral component (36 %, wt/wt) being due to salts present in the mineral medium. A conversion into biomass of the total COD incoming the reactor equal to 29.7 ± 1.2 % COD_{VSS}/totCOD was obtained.

Regarding the composition of fermented products in the effluent, Fig. 3 shows the results in terms of acids production. The total conversion ranged from a minimum of 7 % in the first days of operation to a maximum of 71.8 % at the 73rd day ($COD_{FP}/totCOD_{in}$). Regarding the acids trend, acetic acid was characterized by a constant and slight increase concentration, from 2 % to a maximum of 26 % ($COD_{Ac}/totCOD_{in}$). For propionic and iso-butyric acids, from day 24 there was a drop in the production of the first from almost 23 %–13 % ($COD_{Pr}/totCOD_{in}$), followed by an increase in the second from 2 % to almost 6 % ($COD_{Iso\text{-}but}/totCOD_{in}$). The fermented solution, from day 12th to the end of the operation, was mainly composed of higher concentration in acetic (6.5 ± 0.3 g COD/L), followed by propionic (4.0 ± 0.3 g COD/L), iso-butyric (3.8 ± 0.4 g COD/L), butyric (2.7 ± 0.1 g COD/L), lactate (1.1 ± 0.2 g COD/L) and valeric (1.0 ± 0.2 g COD/L) acids, with a residual sugar presence, a percentage chart of components is reported in Fig. S6. The acid effluent composition presented on COD_{acids} basis, 69 % of acids with odd number C-atoms and 26 % with even number (with the remaining 5 % of lactate). The process was characterised by an average acidification degree equal to 54.0 ± 2.0 % and to 62.0 ± 2.0 % of the total and soluble COD, respectively. In general, the production of propionic acid corresponded to a decrease in iso-butyric one and vice versa. This reciprocal shift between the two acids was supported by metagenomic analysis results (*Section 3.3.4*), considering the type of bacterial family established within the reactor, competitive metabolic pathways was triggered, modulating the activity of *Clostridia* and *Bacteroidetes* genera, from which the production of the two acids was obtained, respectively (Arsenault et al., 2015; Chen et al., 2017). However, due to the normal adaptation of the culture, a continuous and slight change in the fermentation products composition during the working days, can be observed. Despite that, on day 60th, the OLR, NLR and PLR was 3 times higher than normal, changing the effluent composition. The OLR is an important parameter that influences the dynamics of the fermentation, altering the metabolic pathways and giving a different final products distribution**.** As an example, in presence of a high OLR there is a bigger production of acetate and butyrate. In general, during an anaerobic digestion process, these carboxylates are recognized as the indicators of an overloading bioreactor (Wainaina et al., 2020). In this work the higher inlet of OLR and nutrients corresponded to an increase in lactate from 0.9 to 5.3 g $COD_{lactate}$/L, suggesting a metabolic shift towards lactate fermentation, typically activated under high substrate loading or oxygen presence, where facultative anaerobes such as lactic acid bacteria transiently dominate due to their rapid energy-yielding pathways

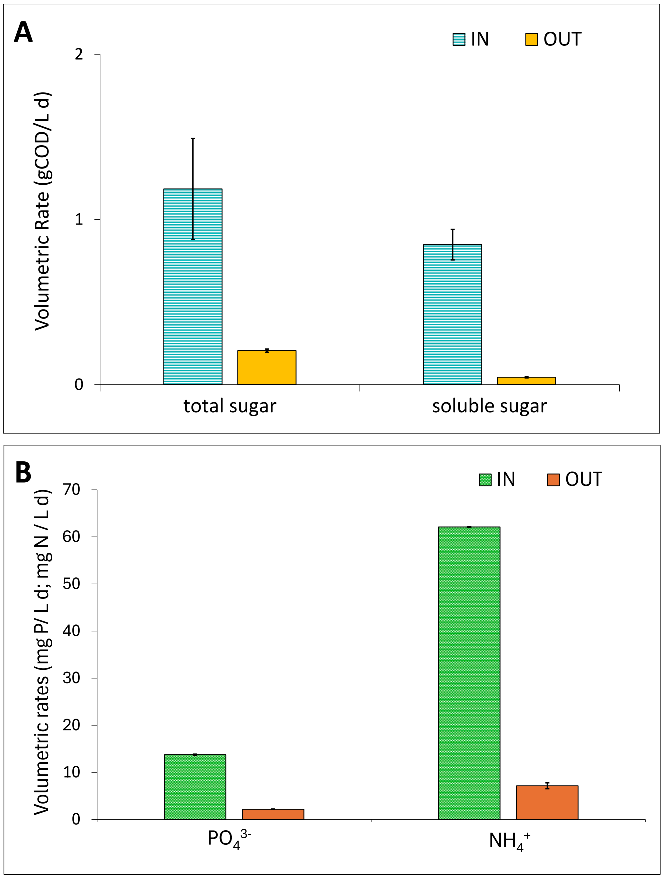

Fig. 2. Characterization of the influent and effluent of the AF reactor: sugars (A) and nutrients (B) volumetric rates.

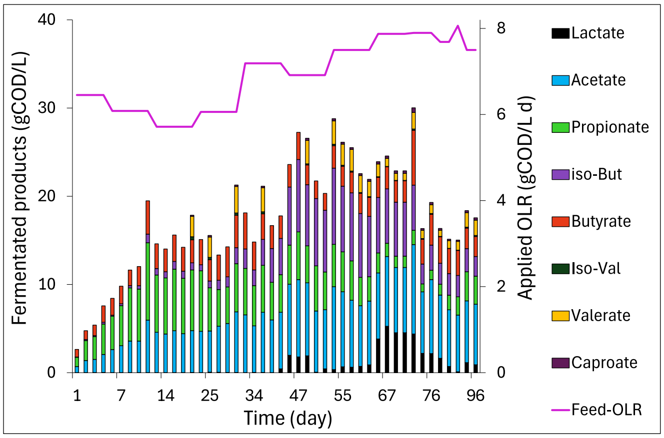

Fig. 3. AF reactor fed with RP solution: trend and concentration of obtained fermentation products along the working days of reactor.

(Versari et al., 1999).

During the 96 days of operation, after an initial biomass acclimatisation phase, the culture produced a fermented solution characterised by a concentration of 18.1 ± 1.0 g COD_{VFAs}/L with a VFA productivity of 3.7 ± 0.3 g COD_{VFAs}/Ld (19.1 ± 1.0 g COD/L concentration and 3.9 ± 0.4 g COD/Ld productivity also considering the lactate). Table S2 compares the results from this study with those from literature. By considering Miranda et al. (2020), where the mixed culture of acetic acid bacteria (AAB) for the fermentation of a carbohydrate rich feedstock (soybean molasses) was used, a lower acid productivity, with a maximum of 0.14 g COD_{acetic}/Ld was obtained, in comparison to the present study. In Ospina-Betancourth et al. (2022), a conversion of the yeast residues (being part of the flour industry) in acids resulted in 50.0 % acidification degree, with a concentration of VFAs in the effluent equal to 2.5 g/L. Also, in a study of Bolaji and Dionisi (2017), the vegetable wastes fermentation in a CSTR achieved an acidification degree of 27 % (on total COD basis), under conditions of neutral pH and 35 °C. Similarly, Dahiya et al. (2015) reported a comparable process efficiency, with 25.3 % degree of acidification ($COD/totCOD_{in}$) of food waste transformed into VFAs during semi-continuous fermentation at pH 10 and 35 °C. Furthermore, Kumar and Mohan (2018), documented that batch fermentation of vegetable waste reached an acidification degree of 54.1 % operating at 28 °C and pH 6. In Greses et al. (2021), where melon and watermelon were selected as a carbohydrate-rich food wastes, with a soluble COD of the used feedstock representing almost 80 % of the total COD, working with a pH between 5.5 and 6.0 and an operative temperature of 25 °C, a fermentation degree of 25.4 % and 25.2 % ($COD_{VFAs}/totCOD_{in}$) were reached for melon and watermelon wastes, respectively. The RP fraction that was unconverted in the acidogenic reactor of the present study (equal to 9.0 % COD/COD) might be due to the presence of RP non-hydrolysable components that are difficult to ferment. Nevertheless, this unconverted fraction is lower than the RP fraction used for bacterial growth and the fraction lost in gas production (*Section 3.2.4*). This result agrees with data previously obtained through batch fermentation tests with RP, in which the maximum conversion into fermentation products on total COD supplied was around 55 ± 2 % (Marchetti et al., 2023). Nevertheless, it can be assumed that, according to the trend of acids leaving the reactor during the 96 days of operation, there was no inhibition of the bacteria by the carbonaceous substrate.

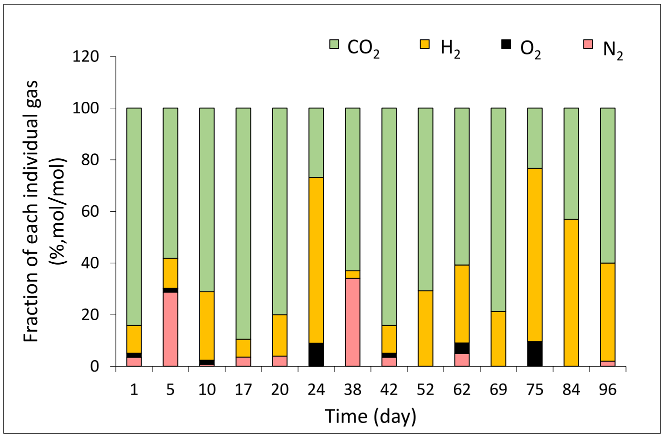

Fig. 4. Characterization of individual gas fraction from AF reactor: trends of fraction of each gas identified in the headspace of acidogenic reactor along the working days of reactor.

3.2.4. *Gaseous products in the AF SBR*

The gas stream leaving the reactor was evaluated, determining the COD converted into it, with the composition trend shown in Fig. 4. Until the 75th operation day, the gas composing was mainly composed by CO_2, which accounted for 70.0 ± 6.0 % of the total produced gas, followed by H_2 (23.0 ± 6.1 %). In the last period from 75th to the 84th day, an increment in the H_2 was detected, with an average value of 62.0 ± 4.0 %. It is worth to mention that no methane production was observed, being a good indication of the occurred acidogenesis and absence of methanogenesis in the system. Regarding the nitrogen gas and oxygen detection, this could be due to sub-optimal isolation of the reactor or due to direct intake of air bubbles during syringe sampling. Moreover, the production of hydrogen can be correlated with the fermentation process. Indeed, the correlation between VFAs and hydrogen production is recognized in the literature, and usually the conditions for which the

Table 3

Heatmap of the most abundant taxonomic groups (≥2 % of abundance in at least one sample) identified in the AF reactor throughout the overall operation. less abundant to more abundant. The obtained data are normalised fraction of the total sequences (%). 🟦⬜🟥

Day 0	Day 20	Day 42	Day 67	Day 96		
0.2	91.3	77.5	80.6	73.2	(C) Bacteroidia (O) Bacteroidales (F) Prevotellaceae (G) Prevotella 7	(P) Bacteroidetes
71.1	0.1	0.0	0.0	0.0	(C) Bacteroidia (O) Bacteroidales (G) OTU_11	
23.4	0.0	0.0	0.0	0.0	(C) Clostridia (O) Clostridiales (F) Clostridiaceae (G) Clostridium sensu stricto 1	(P) Bacillota
0.2	0.0	2.7	4.5	4.8	(C) Clostridia (O) Clostridiales (F) Clostridiaceae (G) Clostridium sensu stricto12	
0.0	2.3	7.1	2.1	13.8	(C) Negativicutes (O) Veillonellales (F) Veillonellaceae (G) Dialister	
0.0	0.8	5.6	4.8	2.7	(C) Bacilli (O) Lactobacillalles (F) Lactobacillaceae (G) Leuconostoc	

P- Phylum; C- Class; O- Order; F- Family; G- Genus best yield of VFAs is obtained corresponds to the lowest yield of hydrogen (Wainaina et al., 2020). For example, in Bouzas et al. (2007), it was found that when the hydrogen partial pressure is high, the fermented conversion does not proceed, due to an inhibition of acetogenic bacteria, causing long chain VFAs accumulation in the system. Usually, this phenomenon is ascribed to potential acid inhibition affecting the hydrogen-producing microbes (Argun and Kargi, 2011; Datar et al., 2007). Therefore, here it is worth noting that the conditions for which there was a lower conversion into fermentation products (Fig. 3, days 75–96) coincided with the higher content of hydrogen from day 75th onward (Fig. 4), where an instability of the system occurred. Nevertheless, along the days of reactor operation, an average amount of 1.62 ± 0.8 g COD_{H2}/d was produced, corresponding to a conversion to hydrogen of 3.9 ± 0.7 % ($COD_{H2}/totCOD_{in}$) of the total COD fed to the reactor. By considering the reactor products in terms of sugars (3.2 ± 0.2 %), fermentation products (54.0 ± 2.0 %), biomass (29.7 ± 1.2 %) and hydrogen (3.9 ± 0.7 %), a conversion around 91 % of the total COD entering in the acidogenic reactor was obtained. This COD balance demonstrates a high level of conversion efficiency and process robustness, reinforcing the feasibility of operating RP-fed acidogenic systems at near-complete substrate valorisation under controlled conditions. The residual 9 % can be related to the non-hydrolysed component present in terms of COD in the effluent.

3.2.5. Microbial community assessment

The analysis of the gene amplicon sequences targeting the bacterial 16S rRNA gene variable region 1–3, combined with taxonomic classification, allowed us to estimate the abundance of different microbial groups. In Table 3 are summarized the 6 most abundant genera identified across all samples collected along the AF reactor operation (from 0 to 96th day) where phylum classification is provided along with genus.

Results showed that along the working period, members of the genus *Prevotella* 7 from the phylum Bacteroidetes became dominant with an abundance ranging from 73.2 % to 91.3 %, followed by organisms from the Bacillota phylum (former Firmicutes). Bacillota are gram positive bacteria, while Bacteroidetes are gram negative, and both substantially contribute to the fermentation of carbohydrates (Stearns et al., 2011). Within this latter phylum, the identified populations belonged mostly to *Prevotella* genus, obligate anaerobic bacteria. *Prevotella*-dominated microbiota is associated with long-term dietary patterns, well known as dietary fiber fermenters, with the former associated with complex carbohydrate consumption. Previous studies have shown that *Prevotella*-dominated microbiota produced 2–3 times more propionate than the other class of Bacteroidetes microbiota (Chen et al., 2017). This would justify the composition of acids leaving the reactor in the first 20 days of operation, where there was a higher production of propionate compared to other acids, in accordance with the highest percentage of this bacterial class on day 20th equal to 91.3 %.

While for Bacillota, the predominant genera were *Dialister*, *Leuconostoc* and *Clostridium*. The latter genus is an extremely active fermentative, characterised by a predominant metabolic pathway leading to the production of butyric acid (Arsenault et al., 2015). Indeed, in accordance with the iso-butyric and butyric acids production in the AF reactor, an increment in the *Clostridium sensu strictu 12* was obtained from day 42nd. Whereas the *Dialister* are not fermentative bacteria (Limeres Posse et al., 2017). They are asaccharolytic bacteria, incapable of breaking down carbohydrates for energy, using amino acids, peptides, and other non-carbohydrate nutrients for growth (Rôças and Siqueira, 2006). The maximum percentage on day 96th, almost 14 %, could indicate an imbalance within the reactor, characterized by a lower output of acid products. However, it was recently seen that *Dialister* bacteria may play a role in lactate or propionate metabolism or participate in syntrophic interactions (Wang et al., 2024). Therefore, its presence does not necessarily imply a negative impact on the overall process.

Indeed, after a temporary decrease in acid productivity (from a maximum of 5.6, in day 73, to 2.8 gCOD/L d), the microbial community exhibited a functional resilience, as shown by the subsequent rise to 3.5 gCOD/L·d by the end of the monitoring period. Lastly, the *Leuconostoc* is generally linked to a particular metabolic pathway that allows to carry out malolactic fermentation (Versari et al., 1999) and from day 42nd, where the presence of *Leuconostoc* was higher (5.6 %), a lactic acid production around 1.7 ± 0.3 g COD/L was observed.

The change in the bacterial population within the reactor can be explained both by natural selection during the working days or also be linked to external factors (reactor imbalance) that may have affected the metagenomic distribution. It reflected the sensitivity of the bacterial population to the changes in reactor operating conditions, adapting to the new circumstances, thus making substantial changes to the trend of acids production. However, to enhance the predominance of the desired bacterial classes, the operating parameters must be varied appropriately. For example, *Clostridium* bacteria can grow in a pH range of 4–5 to 7 and depending on the required composition of the fermentation broth, the pH can be appropriately varied (Zigová and Šturdík, 2000). Hence, if a higher percentage of acetic acid were preferred in the presence of this bacterial class, the pH would have to be adjust to value of 5, as it has been observed that at this pH a metabolic shift associated with the decrease in phosphotransbutyrylase (PTB) activity and the increase in phosphotransacetylase (PTA) activity occurs, leading to a decrease in butyric acid (optimal pH equal to 6) (Zhu and Yang, 2004). This highlights how process conditions such as pH, retention time, feeding strategy can drive the process and being engineered can promote microbial consortia that favour specific fermentation pathways, representing a core element of process customisation in acidogenic systems.

Based on the considerations made so far, the *Prevotella* and *Clostridium* genus would be preferable in an acidogenic fermentation context. While regarding *Prevotella,* it plays an essential role in lignocellulose fermentation processing and, accordingly with other studies, could divert the hydrogen flow in glycolysis, in favour of propionic acid production (Aguilar-Marin et al., 2020; Betancur-Murillo et al., 2022).

4. Conclusions

This study demonstrated the potential of acidogenic fermentation as a sustainable strategy for valorising RP, a carbohydrate-rich by-product of the food industry, performed by MMC consortia. This feedstock showed good solubilization and fermentability properties under semi-continuous operation in a lab-scale sequencing batch reactor. The process achieved a stable acidification degree of approximately 54 % ($COD_{FP}/totCOD_{in}$). The resulting fermented effluent was mainly composed of volatile fatty acids, particularly of 69 % and 26 % on CODacid basis, in terms of VFAs with odd and even C-atoms number, along with minor amounts of lactate (5 %). These compounds are valuable intermediates for a wide range of applications, including the synthesis of high-value platform chemicals. These findings outline a sustainable route of integrating RP valorisation into circular bioeconomy systems, where the fine-tuning of key process parameters enables optimisation of fermentation performance, also laying the basis for process scale-up. Future research will focus on the techno-economic assessment and downstream integration with various biotechnological valorisation pathways, with particular attention to the recovery of the fermented liquid through separation strategies such as centrifugation, membrane processes, or polyelectrolyte-based flocculants for suspended solids removal.

CRediT authorship contribution statement

Angela Marchetti: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Miguel Palhas:** Investigation, Formal analysis. **Marianna Villano:** Writing – review & editing, Supervision, Funding acquisition. **Maria A.M. Reis:** Writing – review & editing, Validation, Funding acquisition. **Joana**

Fradinho: Writing – review & editing, Validation, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2025.146633.

Data availability

Data presented in this study are available upon reasonable request.

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