Innovative co-production of polyhydroxyalkanoates and methane from broken rice

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Abstract

Broken rice, a low-cost starchy residue of the rice industry, can be an interesting substrate to reduce the polyhydroxyalkanoates (PHAs) production cost. However, since the most common PHAs-producing strains lack amylases, this waste must be firstly hydrolysed by additional commercial enzymes. In this work, the acidogenesis phase of the anaerobic digestion was exploited as efficient hydrolysis step to convert broken rice into volatile fatty acids (VFAs) to be used as PHAs carbon source by *Cupriavidus necator* DSM 545, one of the most promising PHAs-producing microbes. Broken rice, both non-hydrolysed and enzymatically hydrolysed, was processed in two continuous stirred tank reactors, at hydraulic retention times (HRT) of 5, 4 and, 3 days, to produce VFAs. The highest VFAs levels were obtained from non-hydrolysed broken rice which was efficiently exploited for PHAs accumulation by *C. necator* DSM 545. Moreover, in view of a biorefinery approach, the residual solid fraction was used for methane production resulting in promising CH_4 levels. The highest PHAs titers and methane yields, 0.95±0.02 g/L and 228.63±20.56 mL CH_4/gVS, respectively, were both achieved for 4 days HRT.

These results demonstrate that broken rice could be efficiently processed into two valuable products without any costly enzymatic pre-treatment and pave the way for future biorefining approaches where this by-product can be converted in a cluster of added-value compounds.

***Keywords*:** starchy organic waste, broken rice, *Cupriavidus necator* DSM 545, anaerobic digestion, biorefinery

1. Introduction

During the last decades, the increase in the fossil-based plastic applications is triggering huge environmental issues, further highlighting the need to investigate new alternatives to replace the fossil-based plastic materials (Prata et al., 2019; Verlinden et al., 2007). The environmental accumulation of plastic waste will reach nearly 12,000 million tons by 2050 (Sheldon and Norton, 2020) leading to additional environmental pollution threats. Nowadays, some bioplastics as poly-lactic acid (PLA), polybutylene succinate (PBS), and polyhydroxyalkanoates (PHAs) are already available. PHAs, a family of biodegradable polyesters (Meereboer et al., 2020), represents a very interesting alternative to the oil-derived plastics because of their chemical-physical characteristics similar to the most common single-use plastics polyethylene (PE) and polypropylene (PP) (Sheldon and Norton, 2020). Despite their great advantages over fossil plastics, PHAs production is still very expensive as 50% of the total cost is linked to the carbon sources, usually glucose or glycerol (Favaro et al., 2019; Koller et al., 2017). In fact, since the most known PHAs-producers lack hydrolytic enzymes, the PHAs accumulation with complex carbon sources required specific pre-treatments or enzymes addition. Therefore, the search for low-cost suitable substrates is crucial for PHAs production at industrial levels and, consequently, for their market competitivity (Akiyama et al., 2003).

The agricultural by-products could be promising PHAs feedstocks. Among all, an interesting substrate is represented by broken rice, a starchy rice milling residue, which accounts for a worldwide availability of about 45 million tons (Favaro et al., 2017). Broken rice was already exploited for fructose syrup (Chen and Chang, 1984), high-protein rice flour (Chen and Chang, 1984), and ethanol (Myburgh et al., 2019).

This substrate was recently converted to PHAs using *Cupriavidus necator* DSM 545 in an SSF (Simultaneous Saccharification and Fermentation) setting (Brojanigo et al., 2020). PHAs content was promising, with a dry cell matter content of 44.09% and a final PHAs concentration of 5.18 g/L. However, to hydrolyse starch in glucose, a costly commercial enzymatic cocktail (STARGEN^{TM} 002) was needed. Therefore, an alternative strategy to avoid the use of the expensive enzymes and/or costly pre-treatments would improve the economic feasibility of the conversion of broken rice, and the other starch-rich materials, into PHAs. This perspective can be achieved by two different paths: i) the development of an engineered starch-hydrolysing *C. necator* strain as recently described by this group (Brojanigo et al., 2021) or ii) the search for low-cost hydrolysis processes. This paper specifically targets the latter strategy towards the conversion of broken rice into PHAs by exploiting the hydrolysis and acidogenesis steps of anaerobic digestion (AD) (Campanaro et al., 2016; Weiland, 2010), as milestones to process starch into volatile fatty acids (VFAs). The liquid fraction of the acidogenesis process was used as carbon source for PHAs accumulation, whereas the solid fraction was exploited in anaerobic batches for methane (CH_4) production. Although other studies are reported in literature on the VFAs conversion into PHAs by the wild-type *C. necator* from cheese whey (Domingos et al., 2018), olive mill waste (Agustín et al., 2015), sugarcane (Dalsasso et al., 2019), and other food waste (Hafuka et al., 2011; Passanha et al., 2013), this is the first report describing both broken rice acidogenesis towards VFAs production and the co-production of two valuable products (PHAs and methane) after the efficient acidogenic pre-treatment of a starchy residue.

2. Materials and methods

2.1 Broken rice, inoculum, and digested biopulp characterisation

Before characterisation, broken rice, supplied by La Pila (Isola della Scala, Verona, Italy), was pre-dried at 60 °C for 48 h and then left to cool down at room temperature. After 24 h, the feedstock was grounded with a hammer mill (1.00 mm screen). Mesophilic inoculum for biochemical methane potential (BMP) experiments was provided from Hashøj full-scale biogas plant, located in Zealand, Denmark. The inoculum was sieved to remove big particles before the BMP assays.

A mixture of digested food waste (hereafter named as biopulp), obtained from a mesophilic reactor in our lab facilities, was used as a start-up inoculum for the acidogenesis reactors because of its high acidogenic capacity since it was already adapted to easily biodegradable compounds which are converted into VFAs.

APHA standard methods (APHA, 2005) were applied for the determination of total solids (TS), volatile solids (VS), ash, chemical oxygen demand (COD), and total nitrogen (TKN) for broken rice, inoculum, and digested biopulp (Table 1).

Broken rice was also analysed, according to the AOAC (Association of Official Analytical Chemists) (Baur and Ensminger, 1977), for starch, protein, hemicellulose, cellulose and lignin content (Table 1).

2.2 Acidogenesis fermentation experiments

Acidogenesis fermentation was performed using two lab-scale continuous stirred tank reactors (CSTRs): in the first reactor, broken rice was used without any treatment (hereafter named as BR) while, in the second reactor, the by-product was previously enzymatically hydrolysed (hereafter named as HBR).

When needed, the enzymatic cocktail STARGEN™ 002, usually adopted in the industrial applications for the mesophilic saccharification of raw starch, was used to hydrolyse broken rice into glucose. This enzymatic blend contains *Aspergillus kawachii* α-amylase expressed in *Trichoderma reesei* and *T. reesei* glucoamylase that works synergistically for the rapidly conversion of raw starch in glucose. The amylolytic blend had a specific gravity and an enzymatic activity of 1.14 g/mL and 570 GAU/g (GAU, glucoamylase unit), respectively. The hydrolysis of broken rice, with STARGEN™ 002, was performed in a Sartorius bioreactor (Sartorius AG, BIOSTAT®) at 55 °C and pH 4 for 24 h. The resulting hydrolysate was then used to feed the relative CSTR reactor. Each reactor, with a working and total volume of 1.8 and 2.0 L, respectively, was equipped with an influent and effluent bottle and the substrate was provided twice a day by a peristaltic pump. To maintain mesophilic conditions (37 °C), a heated jacket equipped with a probe was installed and two magnetic stirrers were present to maintain homogenised both the influent and the reactors.

In both reactors, to acclimate the microbial community of the biopulp, acidogenesis was performed for 3 days only with the inoculum, before starting the feeding with the BR and HBR. Three different hydraulic retention times (HRTs) at 5, 4, and 3 days were investigated using BR and HBR at organic loading (OL) of 20 gVS/L. Each HRT was maintained three times consecutively (hereafter called phase I, II, III of 5, 4, and 3 days HRT), for a total of 36 days of operation (Table S1).

Anaerobic conditions were established by flushing influents, effluents, and reactors with nitrogen for 10 min each. Every day, immediately after sampling, the pH of the effluents was measured by a pH meter (HANNA Instruments, Italia srl). Phase II of 5, 4, and 3 days HRT from BR reactor was selected, among all the collected effluents, as

143 substrates for PHAs and CH_4 production since the second phase (Phase II) represents
144 the intermediate phase of each HRT. Effluents were centrifuged to separate the liquid
145 fraction, used for PHAs production, from the solid fraction which was used later for
146 BMP experiments.
147
148 **2.3 Bacterial strain, culture media, and PHAs fermentations**
149 *C. necator* DSM 545 was provided by DSMZ (Deutsche Sammlung von
150 Mikroorganismen und Zellkulturen, Germany). All media and effluents used during the
151 experiments were autoclaved at 121 °C for 20 min. The strain was plated on nutrient
152 agar containing (g/L): peptone 15, yeast extract 3, NaCl 6, glucose 1, agar 15.
153 *C. necator* DSM 545 was aerobically pre-inoculated at 37 °C (140 rpm) for 24 h in a
154 250 mL flask with 100 mL of DSM81 broth (DSMZ, Germany) containing glucose (30
155 g/L) as carbon source. Before inoculation, cells were collected after centrifugation
156 (5500 rpm for 15 min) and washed twice with sterile NaCl 0.9% (w/v) to remove any
157 trace of glucose that could interfere with the fermentation. Cells were inoculated at an
158 initial optical density (OD_{600nm}) of 0.3 in 250 mL flasks containing 100 mL of the liquid
159 effluent collected from the acidogenesis reactors. At the beginning, all the DSMZ81
160 broth chemicals were added to the effluents before the fermentation. Specific
161 experiments were also performed supplementing the system only with DSMZ81 broth
162 standard vitamin solution. pH was adjusted at 7, which is the optimal pH for *C. necator*
163 DSM 545 growth (Mohd et al., 2012; Wei et al., 2011), adding NaOH 5 M, and flasks
164 were incubated (140 rpm) at 37 °C.
165 Experiments in DSMZ81 broth with glucose having the same carbon molar availability
166 as the selected effluents were included as benchmarks for PHAs fermentation.

Bacterial cells were collected after 72 and 96 h of fermentation, centrifuged (5500 rpm for 15 min) and kept at -80 °C before freeze-drying for PHAs analysis as described below (section 2.6).

All the experiments were carried out in triplicate, standard deviation is also included.

2.4 Biochemical methane potential (BMP) experiments

The theoretical methane potential of broken rice (both BR and HBR) and of the solid fraction of the selected effluents were calculated using their COD levels (Angelidaki et al., 2011).

The theoretical methane potential of broken rice corresponded to 494.16 $mLCH_4/gVS$, obtained by the conversion based on COD/VS ratio. For the three selected BR effluents, the phase II of 5, 4, and 3 days HRT, the theoretical methane potential of the solid fraction of the effluents, were 365.50, 346.58, and 246.00 $mLCH_4/gVS$, respectively.

The effluents collected from the acidogenesis reactor were centrifuged (5500 rpm for 15 min) to separate the solids from the liquid fraction and only the solids were processed as substrate for methanogenesis experiments.

For the BMP set-up, two different organic loadings, 1 and 2 gVS/L, were tested in triplicate using 500 mL bottles. Substrates were mixed with 120 mL of mesophilic inoculum and with the corresponding weight of distilled water required to reach the working volume of 150 mL. To establish anaerobic conditions, both liquid and headspace were flushed with nitrogen for 10 min each. The bottles were immediately sealed with stoppers and aluminium crimps and incubated at 37 °C. Once a day, the bottles were manually shaken to keep the solution well homogenised.

Benchmark experiments, containing only inoculum and distilled water, were performed to calculate only the CH_4 production of the substrates. Values of CH_4 were expressed in $mLCH_4/gVS$.

2.5 Data analysis

One-way analysis of variance followed by Tukey test ($p < 0.05$) was applied to reveal significant differences among the experimental data. OriginPro 9.0.0 SR2 software (OriginLab Corporation, USA) was used to perform statistical analysis.

2.6 Analytical methods

Elemental analysis was carried out for broken rice and the liquid fraction of the acidogenesis effluents using an inductively coupled plasma equipped with an optical emission spectrometry (ICP-OES).

For BMP batches, the concentration of CH_4 was periodically monitored using a micro gas chromatograph (MicroGC) (Agilent 490, Agilent Technologies, Inc, USA) equipped with a thermal conductivity detectors (TCD) and two different capillary columns, one using argon as carrier gas and the other using helium, operating at 145°C, 30 psi and 100°C, 28 psi, respectively. MicroGC values were analysed by SOPRANO software (S.R.A. Instruments).

TVFAs (acetic, butyric, propionic, hexanoic, valeric, iso-butyric, iso-valeric acid) and lactic acid were analysed with a Thermo AI/ AS 1310 Series Autosampler Trace 1300 GC equipped with a flame ionization detector (FID). The initial temperature of the Agilent J&W capillary column was set at 200 °C and helium was the gas carrier.

Each phase of 5, 4 and 3 days HRT were also evaluated in term of bioconversion efficiency (Greses et al., 2020) using the following equation:

$$\% \text{ Bioconversion} = (VFAs_{effluents} / TCOD_{influent}) \bullet 100$$

where the $VFAs_{effluents}$ is the concentration of the acetic, propionic, isobutyric, butyric, caproic, isovaleric and valeric acid in the effluent measured as g COD/L, and the $TCOD_{influent}$ is the total COD (g/L) of the broken rice used as substrate.

The 3-hydroxybutyric acids (3HB) and 3-hydroxyvaleric acid (3HV) content in microbial cells were quantified according to Braunegg et al. (1978) using a Thermo Finnigan Trace gas chromatograph (GC). GC was equipped with an AT-WAX column (30 m × 0.25 mm × 0.25 μm) and an FID detector. FID was set at 270 °C whereas 150 °C was the temperature of the oven. Helium was the gas carrier with 1.2 mL/min of flow rate and the split/splitless was set up at 250 °C. Benzoic acid, 3HB, and poly 3(hydroxybutyric acid-*co*-hydroxyvaleric acid P3(HB-*co*-12 mol% HV) were used as internal and external standards, respectively.

PHAs were expressed as grams of PHAs per liter of culture or as a percentage of PHAs on cell dry matter (CDM).

3. Results and discussion

3.1 Characterisation of the feedstock

The composition of broken rice (Table 1) agrees with recently reported values. For instance, a cluster of Italian rice varieties was described for similar Mg^{2+} and K^{+} concentrations (Somella et al., 2013). The main component is represented by starch (77.74% TS), followed by protein (8.31% TS), with values consistent with those previously described (Brojanigo et al., 2020; Favaro et al., 2017; Nunes et al., 2017).

3.2 Acidogenesis fermentation profiles

To process broken rice into PHAs by non-amylolytic PHAs producers, the substrate needs to be hydrolysed into glucose by expensive commercial enzymes. These and other pre-treatments costs could be avoided by exploiting microbial acidogenesis under anaerobic conditions, which usually converts carbohydrates, proteins, and lipids in VFAs (Liang and McDonald, 2015; Lu et al., 2020).

In this work, two lab-scale CSTRs were operated for 36 days with an organic loading of 20 gVS/L of BR or, as a benchmark, 20 gVS/L of enzymatically HBR.

During the first three days, to acclimatise the microbial community before starting HRT configurations, the reactors were fed only with mesophilic digested biopulp. Three different HRTs were tested for three phases consecutively: the first HRT was set at 5 days (from day 1 to 15), the second at 4 days (from 16 to 27), and the last at 3 days (Fig. 1). The change in HRT from 5 to 3 days, increased the organic loading rate (OLR) from 4.00 to 6.66 gVS/L per day.

Since pH values affect the hydrolysis of the substrates as well as the VFAs composition and production during the acidogenesis (Lu et al., 2020), pH values were daily monitored (Fig. 1). At the beginning of fermentation, the pH values were similar (pH 4.98), as the same digested biopulp was used for both reactors. Nevertheless, after 36 days, pH values significantly decreased ($p<0.05$) to 3.48 and 2.98 in both BR (Fig. 1a) and HBR reactor (Fig. 1b), respectively. The difference in pH values from the reactors could be due to the lower starting pH of the HBR, which was set at pH 4 to support enzymatic activity of the STARGEN^{TM} 002.

VFAs and lactic acid concentration trends were different in the two reactors (Fig. 2). In both CSTRs settings, acetic, butyric and propionic acid were the main VFAs produced during the acidogenesis (Fig. 2), whereas also small titers of valeric, hexanoic, iso-butyric and iso-valeric acid were detected (data not shown).

These findings agree with other studies reporting that acetic, butyric and propionic acid are the main VFAs produced during the acidogenesis of a carbohydrate-rich substrate (Alibardi and Cossu, 2016; Parawira et al., 2004). In this study, the different VFAs concentrations and profiles here found could be due to the specific operation conditions (*i.e.*, HRT, temperature, and feed) adopted that influenced the metabolic pathways of the established microorganisms during the fermentation (Magdalena et al., 2019; Sarker at al., 2019; Strazzera et al., 2018).

At the beginning of 5 days HRT, the high VFAs concentration (11.13 g/L) is likely derived from the initial biopulp digestion and significantly decreased ($p<0.05$) to 6.31 and 5.43 g/L for BR and HBR, respectively, after the first phase of 5 days HRT due to the feeding with the substrates. In the BR reactor (Fig. 2a), at the beginning of 4 days HRT (day 16), the VFAs daily production was significantly stable at around 4.50 g/L ($p>0.05$). In the HBR reactor, VFAs content was lower with no significant variations ($p>0.05$). This lower VFAs production could be explained by the inhibition in acidogenic bacteria occurring in the inoculum as the substrate was already hydrolysed and pH was strongly acidic (pH 3). In fact, the optimum pH range for VFAs production goes from 5 to 11, and extremely acidic or alkaline pH values could reduce their production (Dahiya et al., 2015; Singhania et al., 2013). Moreover, as shown in Figure 2b, consistent production of lactic acid was detected indicating a possible lactic acid bacteria proliferation in the reactor with HBR, probably due to the different microbial

284 consortia established as this substrate was previously hydrolysed. This hypothesis is
285 currently under evaluation through Next-Generation Sequencing (NGS) approaches to
286 identify which bacterial families were mostly involved in the acidogenesis of both BR
287 and HBR. Noteworthy, such investigation will be crucial to elucidate how the
288 hydrolysis of the broken rice, before the acidogenesis, differentially shaped the
289 microbial community of the inoculum, resulting in differential metabolites production.
290 The bioconversion efficiency was also calculated for each phase and HRT tested in BR
291 and HBR reactors during the acidogenesis step (Table 2). Higher bioconversion VFAs
292 potential was assessed using BR compared to the HBR performances. The higher value
293 was reached for the 5 days HRT, with an average bioconversion efficiency from the
294 three phases of 33.04% in the case of BR. Bioconversion efficiencies obtained in this
295 work are promising and comparable with values recently reported in the literature
296 (Bolaji and Dionisi, 2017; Inglesias et al., 2019; Greses et al. 2020; Valentino et al.,
297 2018). Greses et al. (2020), using vegetables waste, described bioconversion yields
298 ranging from nearly 40 to 52%, whereas lower bioconversion values of 22 and 31%
299 were found from sewage sludge (Inglesias et al., 2019) and organic waste (Valentino et
300 al., 2018), respectively.
301 Overall, the most efficient VFAs production and bioconversion yields were achieved in
302 the BR reactor. The phase II was then selected as a representative of 5, 4, and 3 days
303 HRT for BMP and PHAs production.
304
305 **3.3 Accumulation of PHAs using the acidogenesis effluents as a carbon source**
306 *C. necator* DSM 545, a well-known PHAs producer, was adopted in a one-step PHAs
307 production process using the VFAs obtained by the acidogenesis of BR, which showed

308 the highest and most interesting VFAs profiles.

309 In the first experiments carried out with the addition of DSMZ81 broth chemicals, *C.*
310 *necator* DSM 545 was able to grow and accumulate PHAs in all the selected effluents
311 (Table 3). PHAs concentrations significantly increase ($p<0.05$) from phase II of 5 days
312 HRT to 3 days HRT with the highest values obtained using phase II of 3 days HRT,
313 with 0.92±0.02 and 0.73±0.04 g/L after 72 and 96 h of fermentation, respectively.
314 Noteworthy, as reported in Table 3, PHAs contained both 3HB and 3HV units probably
315 due to the presence of VFAs with an odd number of carbons, such as propionic and
316 valeric acid, which act as precursors for the synthesis of P3(HB-*co*-HV) (Gahlawat and
317 Soni, 2017). Such co-polymers greatly enlarge the range of applications of the PHAs
318 obtained in this study (Grigore et al., 2019).

319 Biomass and PHAs produced by *C. necator* DSM 545 from glucose, supplemented at
320 levels equivalent on a carbon molar basis to the VFAs available in each effluent, were
321 also quantified (Table 3). Only in the case of 5 days HRT, higher growth and PHAs
322 accumulation was detected with glucose ($p<0.05$), whereas for 4 days HRT glucose
323 supported higher biomass but PHAs accumulation similar to those obtained by the
324 corresponding effluents. Comparable biomass and PHAs values were displayed by *C.*
325 *necator* DSM 545 for 3 days HRT both with effluents and glucose equivalent.

326 Overall, considering the biomass yields and PHAs titers obtained from effluents it
327 seems that the strain was pushed to grow instead of accumulating PHAs. The elemental
328 analysis, conducted for all the three selected effluents, revealed high concentrations of
329 nutrients, and among all nitrogen, mostly in the case of phase II of 5 days HRT (Table
330 4). This could indicate that the digested biopulp, used as a reactor start-up inoculum,
331 initially provided a high nutrients concentration (Tsapekos et al., 2019; Zha et al.,

2020). Such high nutrients content originating from the liquid acidogenesis effluent of the phase II of 5 days HRT, together with the addition of DSMZ81 broth chemicals, may have negatively affected *C. necator* DSM 545 growth and, mostly, PHAs accumulation, which was found to be very low (Table 3). In fact, to trigger PHAs accumulation in *C. necator* DSM 545, unbalance growth conditions should occur, with a C/N higher than 20 (Lee et al., 1994; Obruca et al., 2018). From Table 4, it is evident that unbalanced nutrient conditions were not established during the growth of *C. necator* DSM 545 in the presence of DSMZ81 broth as the C/N values of the three effluents (8.13, 28.99, and 24.61) greatly decreased to 5.02, 7.81 and 7.07 from phase II of 5, 4 and 3 days HRT, respectively, after the supplementation of 1510 mg/L of N according to the formulation of DSMZ81 medium. As such, *C. necator* DSM 545 was stimulated to grow rather than producing PHAs. The monitoring of acids consumptions at the end of 72 and 96 h of incubation (Fig. 3) could explain the different biomass and PHAs patterns exhibited by the strain in the presence of the three HRT effluents. The higher VFAs and lactic acid detected after the fermentation by *C. necator* DSM 545 with the 5 days HRT effluent could be harmful to the bacterial growth and, consequently, limited biomass production and no accumulation was detected (Table 3). Dalsasso et al. (2019) observed a reduction of *C. necator* DSM 545 growth when both acetic and lactic acid were present leading to a rapid consumption of lactic acid at the expense of acetic acid. This agrees with the VFAs consumption reported in Figure 3 for the phase II of 5 days HRT and the resulting low biomass produced by *C. necator* DSM 545 (Table 3). On the contrary, in the effluents from phase II of 4 and 3 days HRT, where lactic acid was absent, VFAs were almost completely depleted by *C. necator* DSM 545, thus further supporting biomass yield (Table 3).

In order to increase the C/N ratio, hence supporting PHAs accumulation in *C. necator* DSM 545, a new set of experiments was carried out using the same effluents without the addition of DSMZ81 broth chemicals, except for standard vitamin solution (Table 3). For phase II of 5 days HRT, PHAs values were still low and comparable with those obtained in the presence of DSMZ81 broth, whereas the biomass slightly increased pointing out that the addition of chemicals may have triggered toxic levels of few nutrients. Supplementing the effluents only with vitamins resulted in significantly higher differences ($p<0.05$) for PHAs titers from phase II of 4 days HRT with 0.95±0.02 g/L of PHAs and 76.55±0.81% on CDM (Table 3).

Noteworthy, the percentage of PHAs on cell dry matter using the effluents from phase II of 4 and 3 days HRT was around 2.7-fold that obtained in the presence of DSMZ81 broth, while the biomass values obtained decreased to about 40% of those detected supplementing DSMZ81 broth. These findings are very promising as higher PHAs levels were obtained without the supplementation of costly chemicals. PHAs titers obtained in this paper could be further improved by the continuous fermentation as well as *C. necator* DSM 545 pre-adaptation to high VFAs concentrations.

The PHAs results obtained by *C. necator* DSM 545 are even of greater value once compared to the low performances reported about the valorisation of starchy wastes to PHAs after their conversion into VFAs. In the literature, there are few examples of starch by-products being investigated as PHAs feedstocks. Yu et al. (2001) processed starchy wastewater into PHAs with an accumulation of about 34.1% on CDM. In their work, the starch-rich wastewater was first converted, at thermophilic temperatures, into VFAs and then the resulting VFAs adopted for PHAs production by *C. necator*. However, the PHAs accumulation was lower (1.2 g/L) compared with the PHAs

accumulation obtained in this work, maybe due to the lower time of fermentation (48 h). Other papers reported the PHAs production by *C. necator* using starchy waste without their previously conversion into VFAs, but in those cases many pre-treatments and/or costly enzymatic steps were required to obtain limited amounts of PHAs, 5.00 g/L (Rusendi and Sheppard, 1995) and 0.61 g/L (Ugwu et al., 2012) both at bioreactor scale.

3.4 Methane production

Besides PHAs, this work focused also on the production of methane from broken rice. BMP experiments were carried out with two different organic loadings (1 and 2 gVS/L) for BR and HBR substrates (Fig. 4). The highest methane yield was obtained for BR with 465.28±47.80 and 493.27±18.34 $mLCH_4/gVS$ at 1 and 2 OL gVS/L, respectively, corresponding to 94 and 99% of the theoretical methane potential yield. Duan et al., (2019) reported that higher CH_4 yield occurs with the increasing of OL.

Also, for HBR, whose starch was already pre-treated by STARGEN^{TM} 002, the highest methane level (478.43±13.71 $mLCH_4/gVS$) was achieved with 2 OL gVS/L (with almost 96% of the theoretical yield) whereas, with 1 OL gVS/L, the substrate conversion was 92% of the theoretical (455.65±21.53 $mLCH_4/gVS$).

BMP batches were then performed on the solids of the effluents collected from the phase II of 5, 4, and 3 days HRT of the BR reactor (Fig. 4). To our knowledge, this is the first time that BMP was performed after acidogenesis treatment on a starchy waste, such as broken rice. This data set will provide useful information towards the development of a biorefinery approach tailored to the production of a cluster of bioproducts from a single feedstock. As reported in Figure 4, with 1 OL gVS/L, the methane yield was 134.89±28.44, 192.15±25.44, and 90.79±6.48 $mLCH_4/gVS$ for 5, 4,

and 3 days HRT, respectively. Increasing the organic loading at 2 gVS/L, also methane values from the solid effluents increased with 168.01±7.65, 228.63±20.56, and 102.73±7.55 mLCH_4/gVS for 5, 4, and 3 days HRT, respectively, following the same increasing trend of the initial, BR and HBR, substrates with the higher CH_4 levels achieved at 2 gVS/L. Significant differences between the three solid fraction of the effluents tested and the two OLs selected were recorded ($p<0.05$). Therefore, the highest CH_4 yield was achieved from the solids of phase II of 4 days at 2 OL gVS/L. As expected, the solid fraction of BR collected effluents gave CH_4 levels much lower than those reported for the initially broken rice feedstock, since the rest of the organic material dissolved in the liquid fraction was reserved for VFAs-to-PHAs conversion. This hypothesis is in agreement with the different COD values analysed for the substrates: 392.48, 255.99, 309.91, and 268.3 mg/L for broken rice, 5, 4, and 3 days HRT, respectively, with a much lower organic content detected in the case of the effluents due to their partial conversion into VFAs during the acidogenesis step.

4. Conclusions

This research paves the way for the processing of broken rice in two valuable products: PHAs and CH_4. Non-hydrolysed broken rice was effectively converted into high VFAs levels through the acidogenesis step. The spent solids were then processed into CH_4 whereas the liquid fraction was efficiently converted into PHAs by *C. necator* DSM 545. Liquid and solid effluents of 4 days HRT displayed the highest PHAs and CH_4 values. Techno-economical evaluations are in progress to assess the overall feasibility of the process, in view of supporting the definition of biorefinery approaches converting organic waste into clusters of valuable compounds.

Credit Author Statement

Silvia Brojanigo: Participating in Conceptualization and Methodology, Investigation, Data curation, Writing original draft, Visualization. **Merlin Alvarado-Morales:** Participating in Conceptualization and Methodology, Data curation, Participating in Writing original draft, Visualization. **Marina Basaglia**: Commenting revised draft, Funding acquisition. **Sergio Casella:** Commenting revised draft, Funding acquisition. **Lorenzo Favaro:** Conceptualization, Methodology, Data curation, Reviewing original draft, Editing, Visualization, Supervision, Funding acquisition. **Irini Angelidaki**: Participating in Conceptualization and Methodology, Commenting revised draft, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal views that have appeared to influence the work described in this manuscript.

Acknowledgments

This work was funded by Università degli Studi di Padova through BIRD210708/21, DOR1928058/19, DOR2084579/20, DOR208705/20, DOR2107797/21, DOR2114239/21. The Authors are very grateful to Valentino Pizzocchero (MSc, Università degli Studi di Padova) for valuable support in HPLC and GC analyses.

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Figures legends (no print colours)

Figure 1: Profiles of pH and total volatile fatty acids (TVFAs) during 36 days acidogenesis of non-hydrolysed broken rice (a) and hydrolysed broken rice (b).

Figure 2: Total volatile fatty acids (TVFAs), acetic, butyric, propionic and lactic acid profile during 36 days acidogenesis of non-hydrolysed broken rice (a) and hydrolysed broken rice (b).

Figure 3: VFAs (acetic, butyric, iso-butyric, hexanoic, propionic, valeric and iso-valeric acid) and lactic acid consumption by *C. necator* DSM 545 after 72 and 96 h of fermentation in BR acidogenesis reactor effluents from phase II of 5, 4, and 3 day HRT.

Fig. 4: Methane yield of non-hydrolysed (BR) and hydrolysed broken rice (HBR) substrates and for the phase II of 5, 4, and 3 days HRT from BR acidogenesis reactor. Substrates were loaded at 1 and 2 gVS/L and BMP performed at 37° C.

668 **Table 1**: Characterisation of mesophilic inoculum used for BMP experiments, the
669 digested biopulp, used as inoculum for the acidogenesis reactors, and broken rice (nd:
670 not detected).
671

Parameter	Value	
	Inoculum	**Digested biopulp**
TS (g/100 g)	3.98 ± 0.78	2.80 ± 0.69
VS (g/100 g)	2.43 ± 0.15	2.29 ± 0.56
TKN (g/L)	4.45 ± 0.10	0.30 ± 0.04
COD (g/L)	nd	32.08 ± 1.41
TVFAs (g/L)	nd	11.13 ± 0.77
	Broken rice	
TS (g/100 g)	94.91 ± 0.06	
VS (g/100 g)	93.85 ± 0.10	
Ash (g/100 g)	1.06 ± 0.15	
Protein (% TS)	8.31 ± 0.77	
Starch (% TS)	77.74 ± 5.00	
Cellulose (% TS)	0.22 ± 0.01	
Hemicellulose (% TS)	0.54 ± 0.05	
Lignin (% TS)	-	
TKN (mg/Kg)	15.26 ± 0.02	
COD (mg/Kg)	392.48 ± 0.33	
Ca (mg/Kg)	235.37 ± 29.10	
Fe (mg/Kg)	64.18 ± 1.00	
K (mg/Kg)	1425.28 ± 32.83	
Mg (mg/Kg)	450.13 ± 6.26	
Na (mg/Kg)	140.30 ± 33.99	
P (mg/Kg)	1148.82 ± 25.45	

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Table 2: Bioconversion efficiency (%) of phase I, II and III during the three different HRT at 5, 4 and 3 days.

HRT	Phase	Non-hydrolysed broken rice	Hydrolysed broken rice
5d	I	44.87	42.31
	II	25.90	20.86
	III	28.36	14.18
4d	I	19.53	10.02
	II	18.11	8.16
	III	17.40	7.83
3d	I	17.89	9.12
	II	20.19	6.27
	III	18.48	5.07

689 **Table 3**: PHAs production by *C. necator* DSM 545 after 72 and 96 h growth in BR
690 effluents of phase II of 5, 4, and 3 days HRT. Effluents were supplemented with
691 DSMZ81 broth chemicals, or with vitamin solution, and pH adjusted to 7. Experiments
692 with glucose as the only carbon source, supplemented at levels equivalent on a carbon
693 molar basis to the VFAs available in each effluent, were also performed as a
694 benchmark.

HRT	Substrate	Time (h)	CDM (g/L)	PHAs (%CDM)	3HB (%CDM)	3HV (%CDM)	PHAs (g/L)
5 d	Glucose	72	2.98 ± 0.22	46.46 ± 5.59	46.46	-	1.39 ± 0.22
		96	3.12 ± 0.03	44.96 ± 0.91	44.96	-	1.40 ± 0.02
	DSMZ81	72	0.14 ± 0.24	7.44 ± 2.58	7.19	0.25	0.14 ± 0.10
		96	0.34 ± 0.41	13.48 ± 2.64	12.60	0.88	0.34 ± 0.20
	Vitamins	72	0.70 ± 0.02	11.07 ± 0.87	11.05	0.02	0.08 ± 0.01
		96	0.65 ± 0.02	10.77 ± 0.50	10.77	-	0.07 ± 0.01
4d	Glucose	72	2.40 ± 0.07	33.09 ± 1.68	33.09	-	0.79 ± 0.02
		96	2.38 ± 0.11	28.83 ± 2.44	28.83	-	0.67 ± 0.09
	DSMZ81	72	1.66 ± 0.21	26.26 ± 1.84	25.81	0.45	0.43 ± 0.04
		96	1.88 ± 0.08	27.41 ± 5.15	26.96	0.45	0.51 ± 0.08
	Vitamins	72	1.17 ± 0.17	71.33 ± 6.98	69.79	1.55	0.84 ± 0.20
		96	1.24 ± 0.03	76.55 ± 0.81	74.38	2.16	0.95 ± 0.02
3d	Glucose	72	2.34 ± 0.03	28.95 ± 0.89	28.95	-	0.68 ± 0.02
		96	2.31 ± 0.03	27.42 ± 3.45	27.42	-	0.63 ± 0.08
	DSMZ81	72	2.42 ± 0.18	38.25 ± 2.21	38.23	0.02	0.92 ± 0.02
		96	2.32 ± 0.12	31.48 ± 0.08	31.46	0.02	0.73 ± 0.04
	Vitamins	72	1.23 ± 0.41	61.44 ± 0.85	60.99	0.50	0.75 ± 0.24
		96	0.90 ± 0.05	62.79 ± 0.99	61.59	0.49	0.56 ± 0.04

695 **Table 4**: Elemental analysis of BR effluents from the phase II of 5, 4 and 3 days HRT and C/N values in the effluents after DSMZ81 broth
696 supplementation.
697

HRT	Ca	Fe	K	Mg	Na	P	N	C	C/N	C/N after DSMZ81 broth supplementation
					mg/L					
5d	286.93	8.46	205.00	30.50	111.10	56.05	487.63	3963.45	8.13	5.02
4d	20.66	0.65	63.71	13.51	6.29	20.36	111.64	3236.32	28.99	7.81
3d	5.96	0.08	42.41	14.37	3.44	33.29	122.81	3002.18	24.61	7.07

698

699 **Figure 1**

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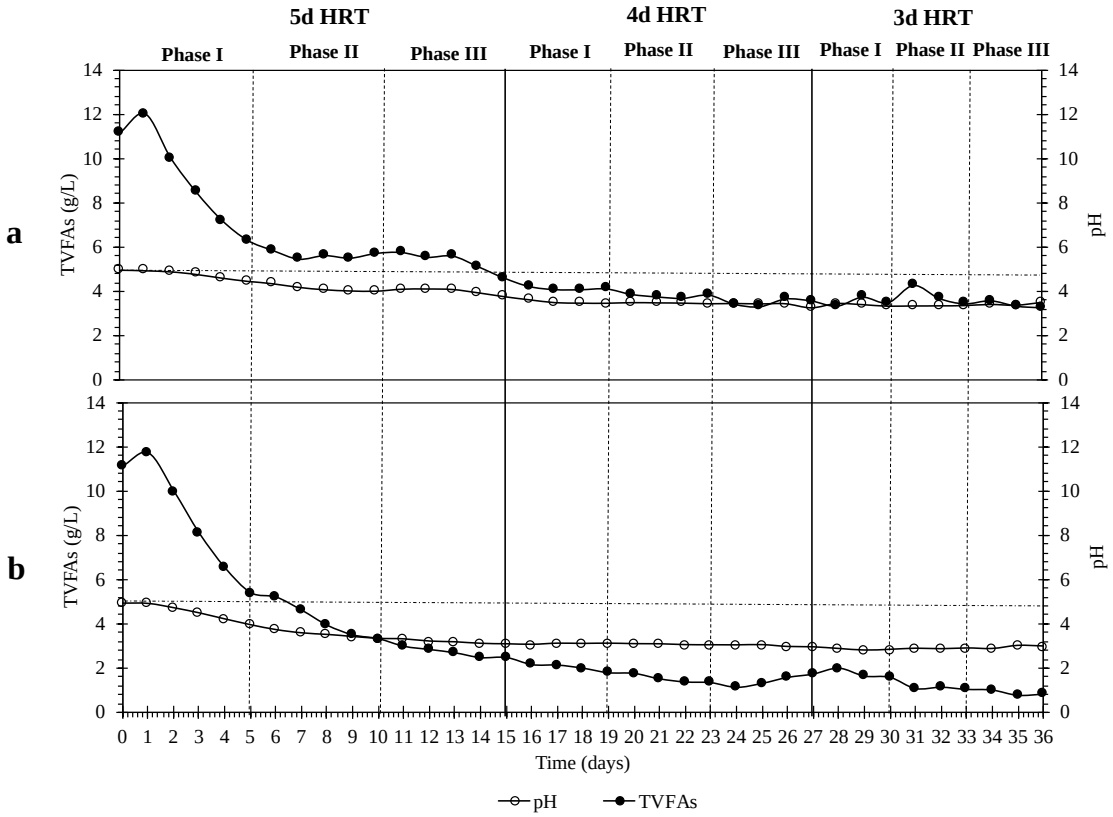

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712 **Figure 2**

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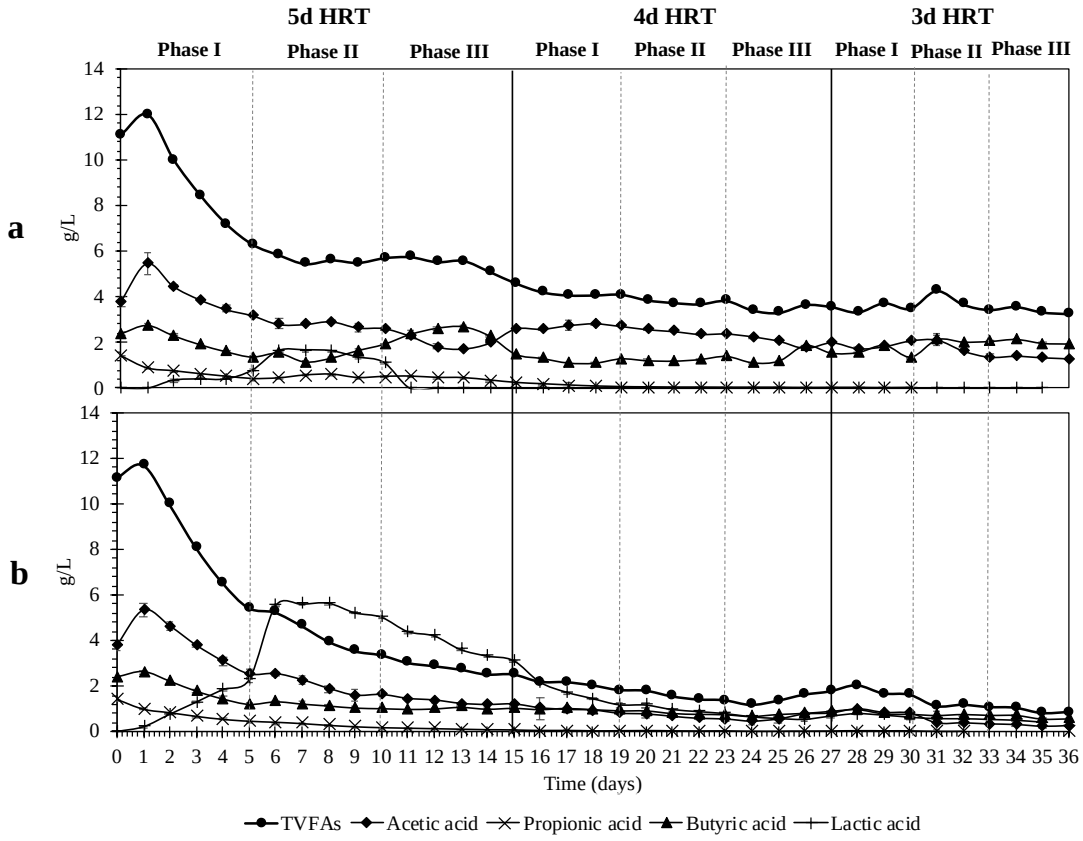

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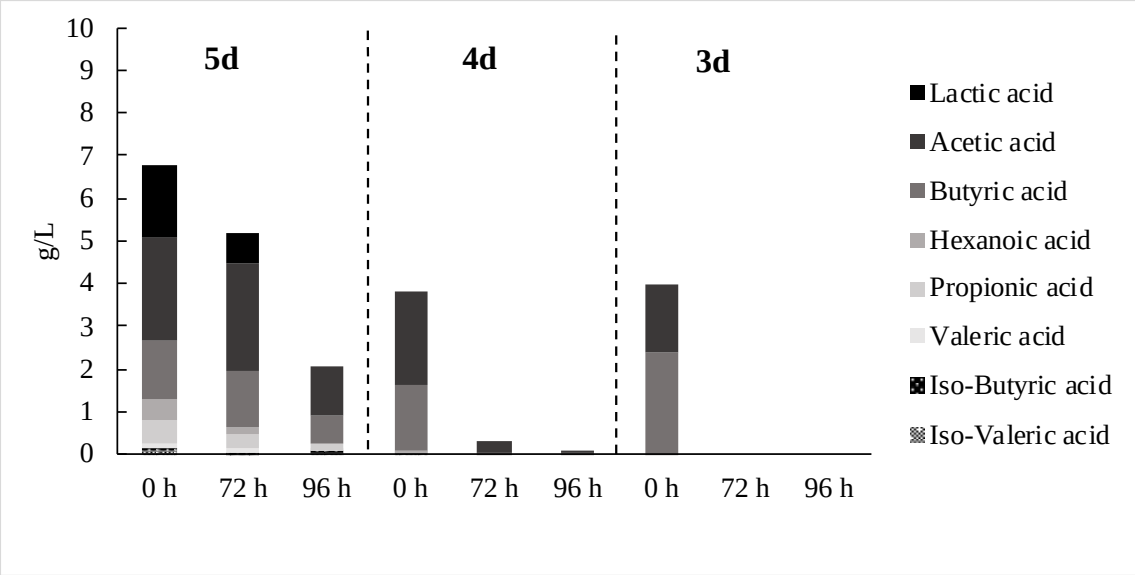
Figure 3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3
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g/L
5d
4d
3d
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72 h
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0 h
72 h
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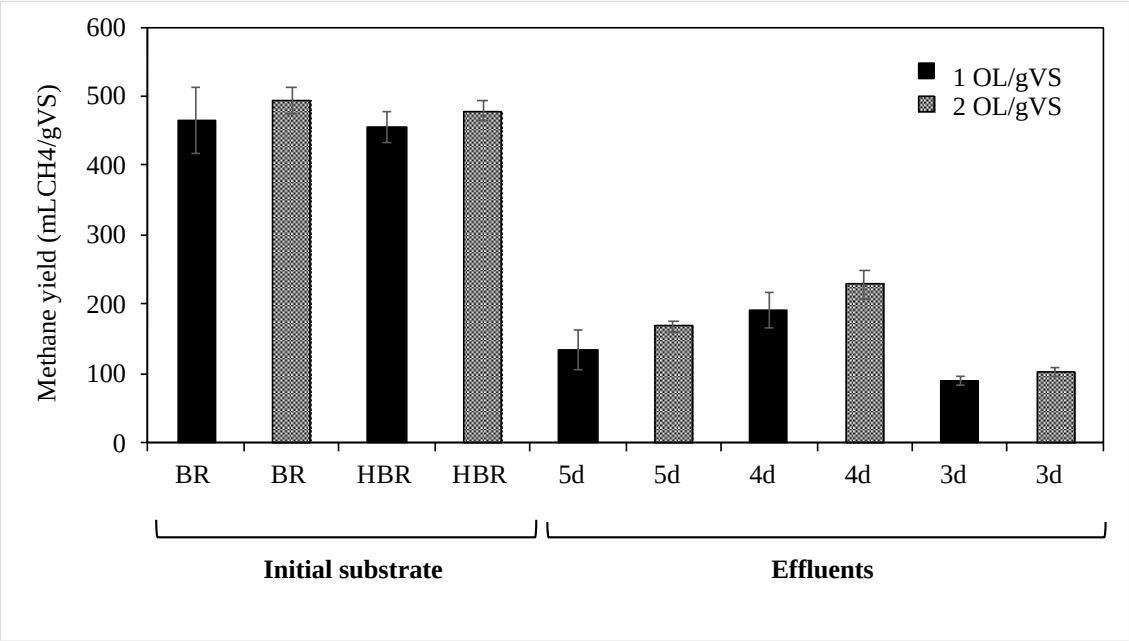
Lactic acid
Acetic acid
Butyric acid
Hexanoic acid
Propionic acid
Valeric acid
Iso-Butyric acid
Iso-Valeric acid

Figure 4


Methane yield (mL CH4/gVS)
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500
400
300
200
100
0
1 OL/gVS
2 OL/gVS
BR
BR
HBR
HBR
5d
5d
4d
4d
3d
3d
Initial substrate
Effluents

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