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**Effect of total solids content on biohydrogen production and lactic acid accumulation
during dark fermentation of organic waste biomass**

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Abstract

Production of biohydrogen and related metabolic by-products was investigated in Solid State Dark Fermentation (SSDF) of food waste (FW) and wheat straw (WS). The effect of the total solids (TS) content and H_2 partial pressure (pp_{H_2}), two of the main operating factors of SSDF, were investigated. Batch tests with FW at 10, 15, 20, 25 and 30 % TS showed considerable effects of the TS on metabolites distribution. H_2 production was strongly inhibited for TS contents higher than 15% with a concomitant accumulation of lactic acid and a decrease in substrate conversion. Varying the pp_{H_2} had no significant effect on the conversion products and overall degradation of FW and WS, suggesting that pp_{H_2} was not the main limiting factor in SSDF. This study showed that the conversion of complex substrates by SSDF depends on the substrate type and is limited by the TS content.

Keywords: Biohydrogen; Lactic acid; Dark fermentation; Total solids; Food waste

Highlights

- H_2 and by-products production were investigated in solid state DF of food waste.
- Effects of TS content and H_2 partial pressure on substrate conversion were studied.
- 15% TS was found to be limiting for H_2 production during solid state dark fermentation.

1. Introduction

Interest in Dark Fermentation (DF) bioprocesses has increased over the past decade because it has shown the potential to produce ‘green’ hydrogen (H_2) from widely available and low-cost organic biomass with a low environmental impact (Azwar et al., 2014). Renewable waste biomass such as agricultural residues, organic fraction of municipal solid waste (OFMSW) and agro-industrial waste might give an additional competitive economic advantage to industrial units treating waste feedstocks by anaerobic digestion for local supply of H_2 (De Gioannis et al., 2013; Guo et al., 2010; Urbaniec and Bakker, 2015). OFMSW and lignocellulosic residues such as wheat and rice straw are potential substrates, as they are abundant and do not compete with food crops as for the production of first generation biofuels (Ghimire et al., 2015a; Guo et al., 2010).

Based on the total solids (TS) content of the digestion mixture, anaerobic fermentation processes such as anaerobic digestion (AD) processes can be categorized as: i) wet AD, which operates at low solids content ($<15\%$ TS) and ii) dry AD at high solids content ($>15\%$ TS) (Motte et al., 2013). Alternatively, AD processes are categorized as: (i) wet ($<10\%$ TS), (ii) semi-dry ($10\text{--}20\%$ TS) and (iii) dry ($>20\%$ TS) (Karthikeyan and Visvanathan, 2012). Dry anaerobic fermentation processes can be operated at high substrate concentrations with little water addition, which offers the advantages such as of: (i) economic benefits by reducing reactor volumes and energy consumption for heating the digester, (ii) more efficient handling of the digestate by reducing the drying post-treatment operation and (iii) a higher simpler technicity (Karthikeyan and Visvanathan, 2012; Motte et al., 2013). Dry AD processes have been successfully applied to many different agricultural feedstocks (Liotta et al., 2015; Mussoline et al., 2012).

Dry ~~AD~~ anaerobic fermentation processes, in particularly dry DF, can also be attractive for commercialization of bio-H₂ processes for the same reasons. Dry DF which operates at a TS content higher than 15% (sometimes also defined as > 20% TS), is also known as Solid State Dark Fermentation (SSDF). SSDF has recently been reported as suitable for biohydrogen production from wheat straw (WS) (Motte et al., 2015, 2014). In their study, Motte et al. (2015) proposed to combine SSDF with a mechanical pre-treatment process to enhance the overall conversion of lignocellulosic biomass, which makes it more feasible for upscaling in a biorefinery concept.

However, increasing the TS content during dry anaerobic fermentation can impact both the total substrate degradation and the biogas production (Abbassi-Guendouz et al., 2012; Motte et al., 2013). At high (19 – 25 %) TS content, mass and energy transfer are lowered by the low water content (Motte et al., 2014; Valdez-Vazquez & Poggi-Varaldo, 2009). These can lead to a local accumulation of by-products up to inhibitory concentrations or result in decreased metabolic conversion rates. Fernández et al. (2008) reported 17% decrease of the degradation rates in dry AD of OFMSW, when the TS content increased from 20 to 30%. Cazier et al. (2015) showed that high hydrogen partial pressures (pp_{H₂}) inhibited ~~wheat straw~~ WS hydrolysis in dry AD, suggesting that local accumulation of metabolic intermediates adversely impacted the overall conversion of the biological process. More particularly, Motte et al. (2013) reported a significant decrease in H₂ production along with a sharp decrease of WS conversion when performing SSDF at 19 % TS. Moreover, a reduction of the H₂ yields and shifts in the microbial community composition towards lactic acid metabolism was observed at high TS content (Motte et al.,

2014). In contrast, only few studies have addressed the effect of increasing the TS content on DF of food waste (Valdez-Vazquez and Poggi-Varaldo, 2009). Therefore, this study aims to investigate the effect of the TS content on organic waste conversion in SSDF using food waste (FW) and Wheat Straw as high and low biodegradable substrate, respectively. In addition, the effect of the pH_2 on organic waste hydrolysis and metabolic pathways was evaluated using SSDF batch tests.

2. Material and Methods

2.1 Substrate feedstock

Batch SSDF experiments were carried out with synthetic FW (20.6% TS and 19.7% VS), freshly prepared with a well-defined composition as described elsewhere (Ghimire et al., 2015b) and similar to the composition of FW in European countries reported by VALORGAS (2010). WS with a TS and VS content of 95% and 97%, respectively, was used as a model lignocellulosic biomass substrate.

2.2 Source of inoculum

Waste activated sludge was used as inoculum after heat-shock treatment (90 °C, 15 min) to enrich spore forming *Clostridium* sp. and inhibit methanogenic and homoacetogenic activities that consume H_2 (Ghimire et al., 2015a). It was sampled from the secondary clarification unit of a municipal wastewater treatment plant located in the center of France with a capacity of 285,000 population equivalents. The waste activated sludge was centrifuged at 6,500 rpm for 20 min (4 °C) to reach 13.9% TS and 9.7% VS content for achieving a higher TS content during all experiments.

2.3 Experimental set-up

2.3.1 Effect of TS content on H₂ production and substrate conversion

Batch SSDF tests were carried out in triplicates at 10%, 15%, 20%, 25% and 30% TS content of FW. In 600 ml flasks, 53.4 g of an inoculated biomass mixture composed of FW (20.3 g wet weight with 19.7% VS) and inoculum (4.1 g wet weight with 9.7% VS) with a substrate (S) to inoculum (X) ratio (S/X) of 10 g VS substrate/g VS inoculum were mixed with 16.0 g of 2-(N-morpholino) ethanesulfonic acid (MES) buffer, 12 ml of 3.2 % NaOH and 1 ml of trace metal solution containing (g/L): FeCl₂ 2 , CoCl₂ 0.5 , MnCl₂ 0.1, NiCl₂ 0.1 , ZnCl₂ 0.05 , H₃BO₃ 0.05 , Na₂SeO₃ 0.05 , CuCl₂ 0.04 and Na₂MoO₄ 0.01 . This mixture had an initial pH of 5.5. The amount of distilled water to be added was calculated with a mass balance on the TS content, including substrate, inoculum, buffer and the other solutions added to obtain final TS contents of 10.0 (± 0.01), 14.98 (± 0.03), 19.89 (± 0.04), 24.92 (± 0.02) and 30.0 (± 0.07) %TS. Batch tests were then incubated at 37 (± 1) °C for 14 days.

2.3.2 Effect of partial pressure of H₂ (pp_{H2}) on substrate conversion

The study on the effect of the partial pressure of H₂ (pp_{H2}) was carried out in four replicated batch tests operated with FW or WS at a S/X ratio of 10 and a final TS content of 25 (± 1) %. A final TS of 25% was selected to ensure the effect of a high TS content on substrate conversion. Tests were carried out with a thin layer of digestate (< 1 cm), approximately 22 (± 2) g, to minimize the effect of gas diffusion (Cazier et al., 2015). H₂ gas was initially added in the headspace of 600 ml serum bottles according to the two following sets of tests: pp_{H2} equivalent to 542 (± 32) mbars (33 ± 2 % H₂ in the headspace, named as “A”) and 1087 (± 29) mbars (66 ± 1 % H₂ in the headspace, named as “B”). A control was carried

out with only N₂ in the headspace (named as “C”). In all tests, the initial total pressure was set at 1,500 mbars by adding N₂ at the start of the experiments for all conditions. The initial pH was adjusted at 5.5 using the MES buffer. Batch tests were incubated at mesophilic temperature (37 ± 1 °C) for two fermentation periods of 14 and 21 days each.

2.4 Analytical methods

Prior to liquid metabolite measurements, 5 g of digestate was diluted in 5 g of deionized water, mixed during 30 minutes, centrifuged at 39,121g during 20 min at 4°C and then filtrated at 0.2 µm with a nylon membrane. The remaining liquid phase was then used to quantify organic acids, other metabolic end-products and soluble sugars. Dark fermentation metabolites were measured at the beginning and the end of each experiment.

Organic acids were quantified with a gas chromatograph (Perkin Clarus 580) using an Elite - FFAP crossbond® carbowax® 15 m column connected to a flame ionization detector at 280°C, as described elsewhere (Cazier et al., 2015). N₂ was used as carrier gas at a flow rate of 6 mL/min. High performance liquid chromatography (HPLC) was used to quantify other metabolites, e.g. lactic acid, alcohols, and soluble sugars. The HPLC chain was composed of an Aminex HPX-87H column (300 mm on 7.8 mm, Bio-rad), a pre-column to filter residues (Micro guard cation H refill cartridges, Bio-rad) and an automatic sampler (Water 717). A diluted sulfuric acid solution of 0.005 M in milliQ water was used as eluent at a flow rate of 0.4 ml/min.

The gas composition was measured with a gas chromatograph (Perkin Clarus 580) equipped with a thermal conductivity detector at 150°C and an injector heated at 250°C and two capillary columns heated at 60°C. The first column corresponded to a RtU bond for CO₂ analysis, while the second column was a RtMolsieve used for the determination of the

O₂, H₂, N₂ and CH₄ concentration as described elsewhere (Cazier et al., 2015). Argon at a pressure of 350 kPa and a flow rate of 31.8 ml/min was used as carrier gas. Gas production was monitored through the increase in gas pressure, which was periodically measured with a digital manometer (2000, Leo2 Keller, Winterthur, Switzerland).

2.5 Microbial diversity analysis

For microbial analysis, 0.2 – 0.3 g of solid samples were collected at the end of the fermentation period of 14 days from the SSDF batch tests operated at different pp_{H2}. PCR based pyrosequencing, described elsewhere by Carrillo-Reyes et al. (2016), was carried out in order to elucidate the classification of the microorganisms present in the SSDF. The samples for microbial analysis were taken at different pp_{H2} during SSDF of FW and WS.

2.6 Data analysis

Substrate degradation was estimated by calculating a theoretical chemical oxygen demand (COD) mass balance. For this, the difference in metabolic end-products (accumulated in both the gaseous and liquid phase) at the initial and final state were compared. Direct measurement of COD was not considered in this study, as suggested by Cazier et al. (2015), since the COD measurements of the complex organic residues, such as lignocellulosic biomass and FW, may vary more than 10 %, which may be higher than the overall substrate degradation during the SSDF process.

Total substrate degradation was estimated from the amount of COD produced during DF of the substrate estimated per kg of TS added initially, and corresponded to:

$$\text{Total Substrate Degradation} = \text{COD of Final State} - \text{COD of Initial State}$$

$$Total\ Substrate\ Degradation = \frac{A_{H_2,f} + A_{met,f} + A_{GC}}{kg\ TS} - \frac{A_{H_2,i} + A_{met,i}}{kg\ TS}$$

Where: $A_{H_2,f}$ is the amount of H_2 remaining at the end in the headspace, $A_{met,f}$ the final amount of metabolites accumulated, A_{GC} the total amount of gas (H_2) sampled for analyses, $A_{H_2,i}$ the initial amount of H_2 added and $A_{met,i}$ the initial amount of metabolites in the medium.

R (OSX version 3.1.3) with the package Rcmdr (OSX version 2.1.7) was used for statistical analysis of the experimental data. The P value was set at 0.05 and the significance of the results was tested with P values of: * < 0.05; ** < 0.01; *** < 0.001; while results were not significantly different when $P > 0.05$. A multivariate analysis by Principal Components was carried out using FactoMineR (version 1.24, more on <http://factominer.free.fr/>), an extension of the R software, to correlate the H_2 production with the substrate degradation and formation of other metabolic by-products (Lê et al., 2008).

3. Results and discussion

3.1 Effect of TS content on H_2 production and substrate conversion

SSDF tests were carried out with FW at different TS contents (Fig. 1). Overall, the H_2 production was strongly impacted by increasing the initial TS content, which is in accordance with earlier studies (Motte et al., 2014; Valdez-Vazquez & Poggi-Varaldo, 2009). Fig. 1a shows the FW degradation after 14 days of incubation as a function of the TS content. Fig. 1b presents the molar yield (mmol/kg TS) of the major metabolic end products. Substrate conversion decreased and biohydrogen production was significantly lowered when the initial TS content increased (Fig.1b). A simultaneous shift of the metabolic pathways was observed at a high TS content. The maximum and minimum

substrate degradation of $134 (\pm 22)$ and $51 (\pm 3)$ g COD/kg TS was achieved at 10 and 30 % TS, respectively. H_2 accumulated in the headspace only at a TS content of 10%. Hydrogen production drastically decreased and lactic acid production was favored at TS contents higher than 10%. Indeed, from 15% TS onwards, the metabolic pathways shifted towards lactic acid conversion, thus explaining the decrease in H_2 production (Fig. 1b). The highest substrate conversion of $2,901 (\pm 143)$ mM metabolites/kg TS was observed at wet conditions of (10% TS) (Fig. 1b), while it was the lowest ($1,435 \pm 13$ mM metabolites/kg TS) at 30% TS (Fig. 1b). As shown in Fig. 1a and b, such adverse effect of the TS content started between 10 and 15%. Motte et al. (2014) showed similar decrease of the hydrogen yields, related to a metabolic shift with an accumulation of lactic acid, but at higher TS content (19%) for wheat straw only. Further narrowing the study of the TS content within the range 10 – 15 % TS or considering the soluble carbohydrates available to microbial degradation could further give new insights on the mechanisms behind this limiting effect of the TS content in SSDF.

In addition, acetate and butyrate were the two of the major as metabolic by-products in at a TS content of 10%, while lactate dominated at higher TS contents. The PCA correlation plot (Fig. 2a) shows that H_2 production correlated only with butyrate production, suggesting that acetate resulted from DF and homoacetogenesis pathways. Theoretically, the presence of acetate and butyrate as metabolic by-products is correlated with hydrogen production pathways (Ghimire et al., 2015b). However, a correlation with butyrate production as shown in this study (Fig. 2a), is consistent with previous observations made by Guo et al. (2014) for solid waste where homoacetogenesis can occur. Interestingly, H_2 production was also linked to the total substrate degradation, while lactate production did not correlate well with the substrate conversion but more with the initial TS content

suggesting an adverse effect of high TS content likely due to the local microbial environment (Fig. 2b). Indeed, the decrease of substrate conversion at increasing TS might be attributed to VFA accumulation, especially lactic acid, decreasing locally the culture pH and inhibiting the hydrolysis process, even though a high amount of MES buffer was added to the medium. Such reaction is very similar to the ensiling process where pH lower than 4.5 are favored to stop the microbial process (Kafle & Kim, 2013). Consistently, Lin et al. (2006) reported that xylose removal decreased from 85% to 37% when the culture pH decreased from 8 to 5. Similarly, Fang and Liu (2002) reported a 10% decrease in glucose degradation when the pH decreased from 5.5 to 4. The low culture pH could also have had an adverse effect on hydrolytic enzymes. Indeed, Veeken et al. (2000) reported a decrease in hydrolysis of complex substrates with a decrease in pH, since hydrolytic enzymes are optimally functional at neutral pH (Parawira et al., 2005). Another explanation for the decrease in substrate degradation at higher TS content could be the local accumulation of H_2 , as reported and hypothesized by Cazier et al. (2015).

3.2 Effect of pp_{H_2} on substrate conversion

As potential inhibitory factor, the effect of the pp_{H_2} on FW and WS degradation at a high TS content (25 %TS) was investigated and different initial pp_{H_2} were tested. Fig. 3a shows the substrate degradation (expressed as g COD/kg TS) from FW after 14 and 21 days of DF at different pp_{H_2} . The level of inhibition of pp_{H_2} on substrate hydrolysis was determined based on the difference in total substrate degradation (Fig. 3a). No significant effect of the initial pp_{H_2} on biomass hydrolysis (ANOVA test, P-values > 0.05) was observed at 25% TS. This was further evident from the metabolic products that accumulated at the end of the

experimental period, which showed no significant shift when changing pp_{H_2} (Fig 3b). Substrate degradation slightly increased with the fermentation time, and lactic acid and ethanol accumulated as major metabolites in all tested pp_{H_2} with FW. Further tests were carried out with WS under similar experimental conditions with the objective to confirm the results obtained with FW. Although the pH did not significantly decrease at the end of the experimental period, Fig. 3 and the ANOVA analysis (P-values > 0.05) show that the pp_{H_2} did not affect the DF of the substrate WS. However, the difference in metabolites accumulated between 14 and 21 d suggests that hydrolysis occurred. Therefore, it seems that some dark fermentative communities present in the SSDF tests investigated with WS were not strongly affected by the presence of H_2 . These results contrast with the study of Cazier et al. (2015) who reported an inhibition by high pp_{H_2} of WS hydrolysis in dry AD, at pH 7. In their work, inhibition was observed at a pp_{H_2} > 742 mbars and degradation of wheat straw decreased from 90 (\pm 10) to 20 (\pm 10) g COD/kg TS in the controls and at a pp_{H_2} of 1,555 mbars, respectively. In contrast, in this study, no evidence of the effect of pp_{H_2} was observed, suggesting the inhibition of the substrate conversion is mainly due to organic acids (especially lactate) accumulation. Although this difference might be attributed to the lower operational pH in the present study (pH 3.7 - 5.5) compared to the dry AD (pH 7 - 8) process of Cazier et al. (2015), the WS degradation in the control tests, i.e. 22.4 (\pm 2) g COD/kg TS (at pH 5.4), was interestingly very similar to the one reported by Cazier et al. (2015), i.e. 20 (\pm 10) g COD/kg TS, when maximum hydrolysis inhibition occurred at a pp_{H_2} of 1,555 mbars at pH 7 - 8. Therefore, this difference could be attributed to the fact that substrate degradation in this study was strongly related to the high content in soluble carbohydrates present in the FW substrate, as reported by Guo et al. (2013), but not to the substrate to be hydrolyzed such as WS.

Moreover, the pH might have affected microbial selection and the related metabolic pathways. Lactic acid or ethanol were not present as major intermediates in the SSDF of WS (Fig. 3c), in contrast to tests with FW (Fig. 3b). This suggests that the microorganisms producing these metabolites are dominant in moisture limiting and nutrient rich environments such as FW, but not in WS (Sikora et al., 2013).

This is in good agreement with the characterization of the microbial communities. The microbial community characterization (Fig. 4) shows that mainly 3 families represented the most abundant OTUs, showing the community present in the SSDF residue after 14 d incubation had a low diversity, although the number of distinct operational taxonomic units (OTUs) was 1127 and 1877 for the SSDF samples of FW and WS, respectively.. The hydrogen (pp_{H_2}) favored the development of a *Lactobacillales* population (Fig. 4a). The high abundance of *Lactobacillales* (Fig. 4) explains the high lactic acid concentration during SSDF of FW (Fig. 3c). Moreover, the high abundance of *Lactobacillales* at T_0 during SSDF of FW (Fig. 4a) compared to WS (Fig. 4b) implies FW as their major source of inoculum. On the other hand, during SSDF of WS, ~~the~~ *Clostridiales* were present abundantly, which agrees with the production of several other metabolites such as acetate, propionate and butyrate. This observation is also in good agreement with Motte et al.(2014) who previously reported that high TS contents impacted the microbial composition through the selection of lactic-acid bacteria from the *Bacilli* class.

The production of organic acids by fermentative conversion processes is commonly significantly affected by the operating parameters such as culture pH, temperature and substrate concentration (Wang et al., 2014, 2015). However, the highest conversion of FW (140.7 ± 23 g COD/kg VS_{added} at initial pH 5.5, 25 % TS, 14 days SRT and 37 °C) in this

study is lower compared to the study by Wang et al. (2015) (799 g COD/kg VS_{added} at controlled pH 6.0, 35 °C) and Wang et al. (2014) (918 g COD/kg VS_{removed} at controlled pH 6.0, 30 °C). This could explain the higher conversion of the waste biomass into fermentative products reported in these two studies, compared to this study where the pH was not controlled and the low pH conditions (3.7 - 5.1) finally inhibited the microbial activity. Similarly, a comparison can be made between the study of Motte et al. (2015) who obtained 6 fold higher organic acids production (140 ± 6 g COD/kg TS_{added} at 23 % TS, 37 °C) at an initial pH 8.5, as compared with this study (22 ± 2 g COD/kg TS_{added} at 25 % TS, 37 °C) at initial pH 5.5. ~~Therefore, it can be concluded that under SSDF, the substrate conversion depends on the culture pH, TS content, substrate type and microbial community.~~

The total substrate conversion under SSDF also depends on the substrate type. FW has a high VS content (21 to 27% VS) and is particularly suitable for a concomitant production of bio-hydrogen and platform molecules such as short-chain volatile fatty acids, lactic acid and / or alcohols (Uçkun Kıran et al., 2015; VALORGAS, 2010). Soluble by-products of DF can be further applied in biological processes for (i) wastewater treatment (Elefsiniotis et al., 2004), (ii) production of platform molecules such as biopolymers (Ntaikou et al., 2009), (iii) micro-algal lipids production (Turon et al., 2015), iv) H₂ production by photo-fermentation, v) feed for microbial electrolysis cells for production of H₂ and other value added chemicals (El Mekawy et al., 2014) or vi) anaerobic digestion for a higher energy recovery, in the form of methane, i.e. both H₂ and CH₄ (Ghimire et al., 2015a).

5. Conclusion

This study highlighted the effects of limiting parameters on substrate conversion during SSDF of FW and WS. The initial TS content significantly affected the substrate degradation and metabolic by-products during SSDF of FW. The H₂ production ceased at a TS content higher than 15%, accompanied by the accumulation of lactic acid. Moreover, the pp_{H2} did not affect the SSDF of food waste due to lactic acid production lowering the culture pH, which played a major role in limiting the substrate conversion, as confirmed during SSDF of WS.

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Figure Captions

Fig. 1. Effect of TS content on substrate degradation (in g COD per kg of initial TS) (a); and accumulation of end metabolites accumulation (mM per kg of initial TS) (b); at different TS content

Fig. 2. Principal component analysis correlation circle plots: (a) Hydrogen and major metabolic by-products production and (b) substrate degradation and metabolic products

Fig. 3. Substrate degradation (in g COD per kg of initial TS) at 25% TS using FW and WS (a) as well as end metabolites accumulation (mM per kg of initial TS) in SSDF of FW (b) and WS (c) at different pp_{H_2} and at the end of two fermentation times (14 d and 21 d). A = 542 (\pm 32) mbars, B= 1087 (\pm 30) mbars, C = 0 mbars, 14 = 14 d and 21 = 21 d

Fig. 4. Microbial community distribution at family level of the sequenced samples during the SSDF using FW (a) and WS (b) at the beginning (To) and end of the 14 d incubation at different pp_{H_2} (0, 542 (\pm 32) mbars, and 1087 (\pm 30) mbars) considering most abundant OTUs (only OTUs with a presence of >1.0% were considered)

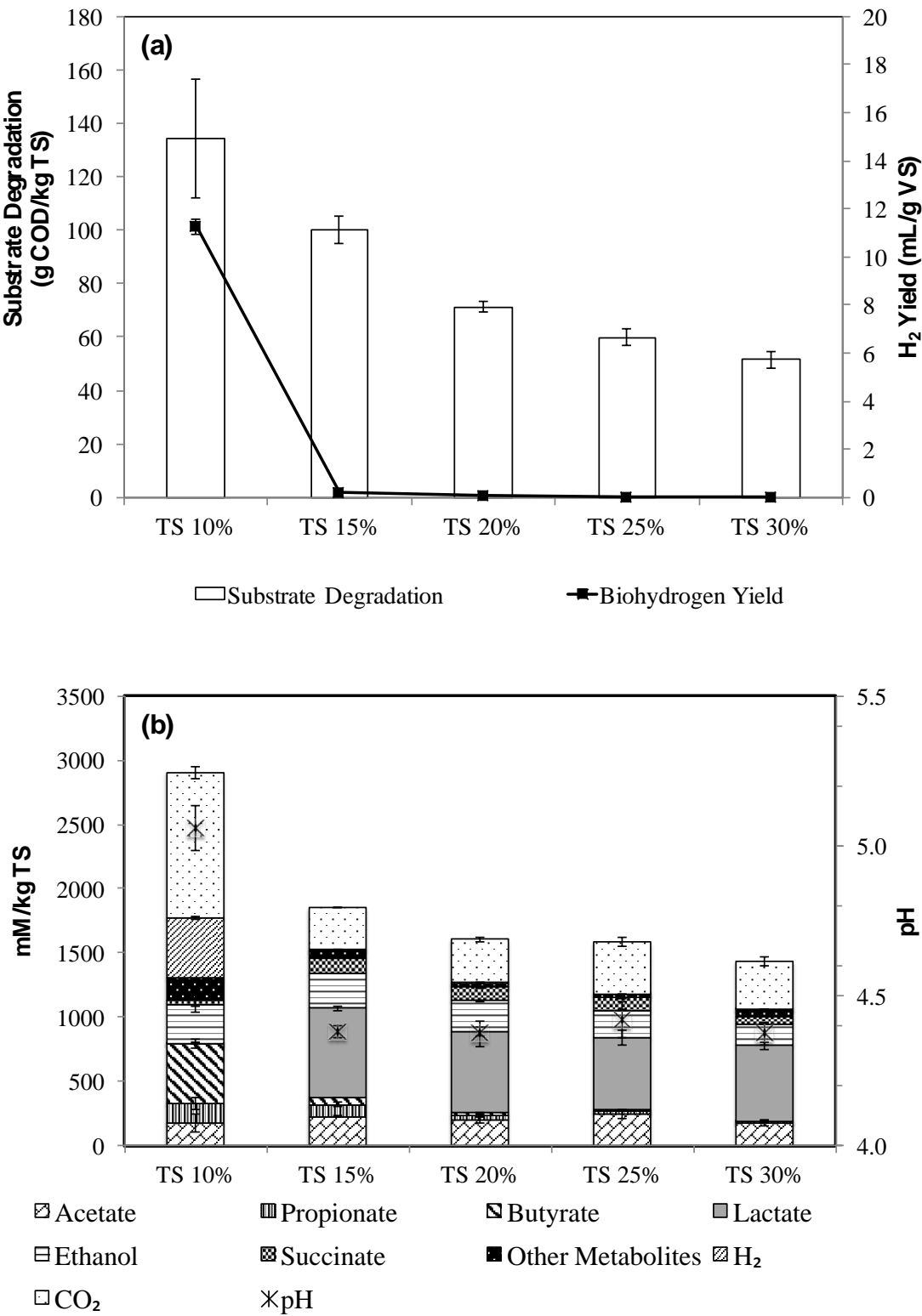
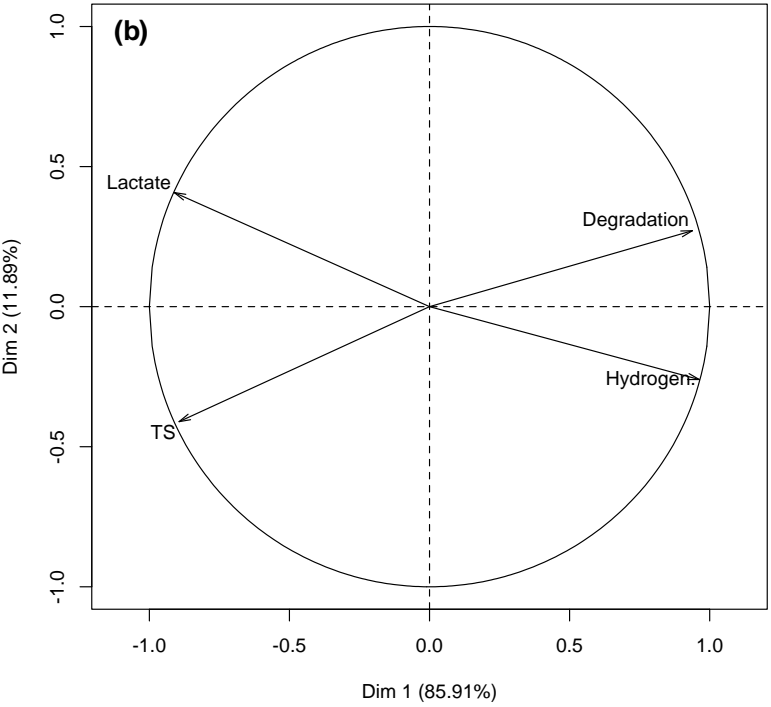
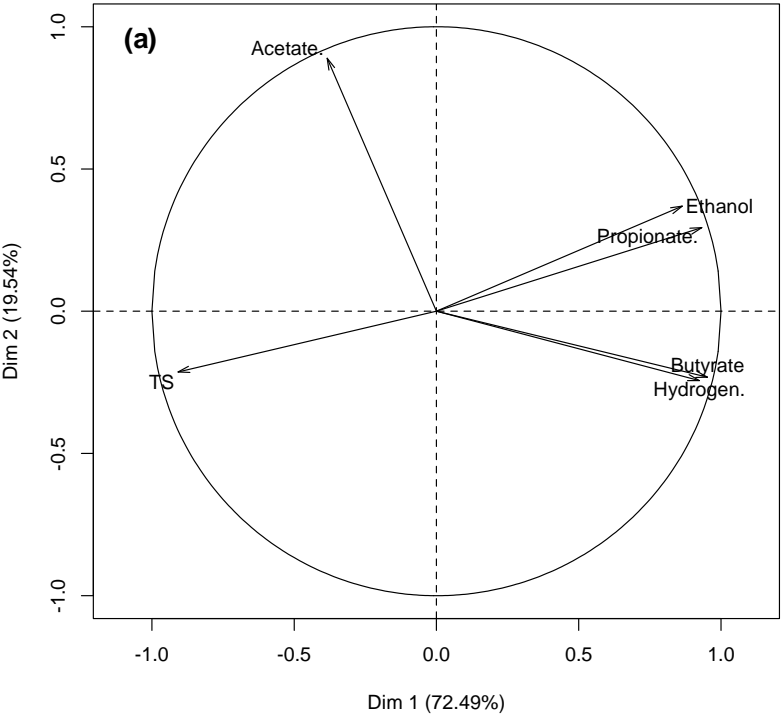
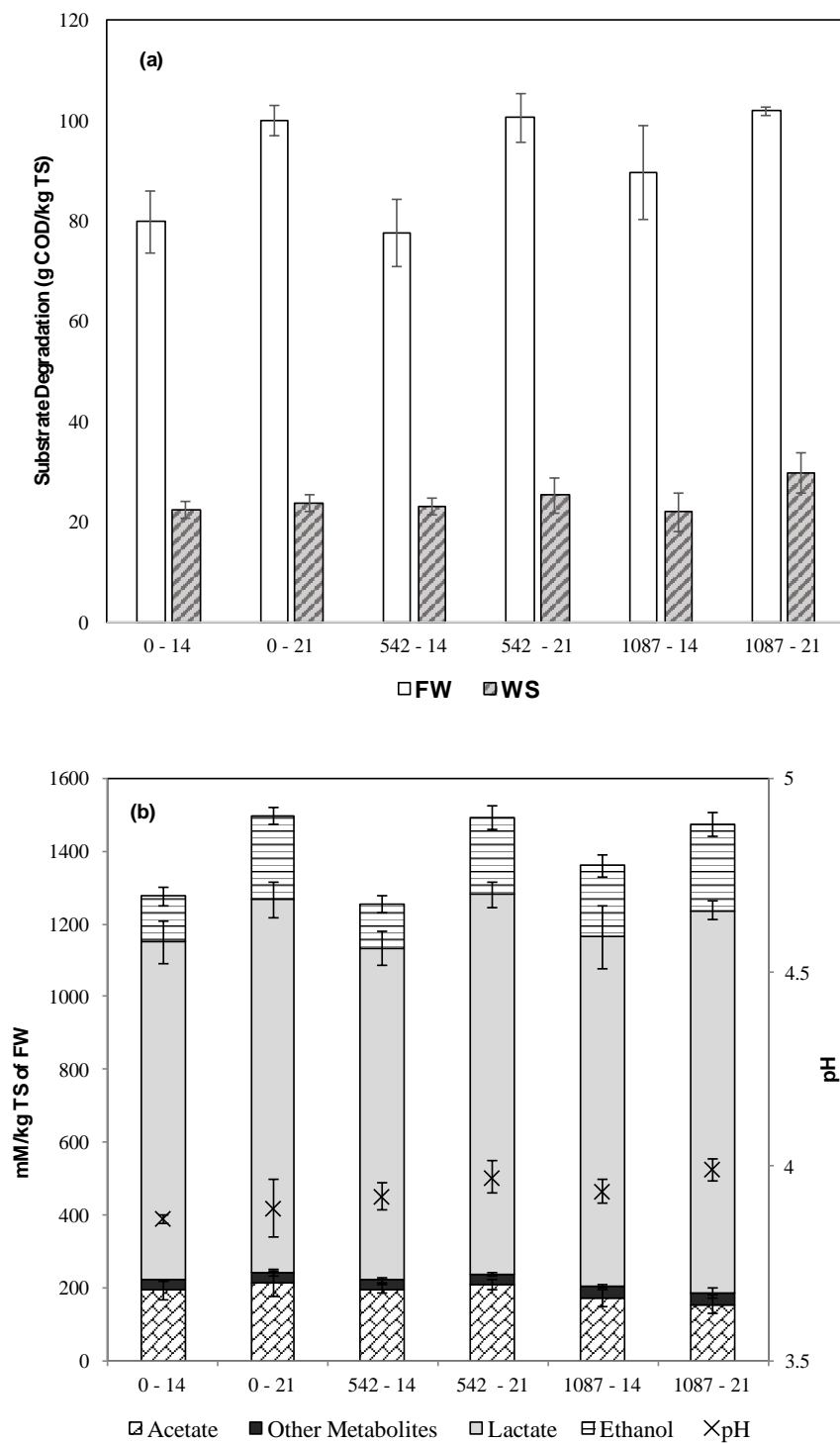
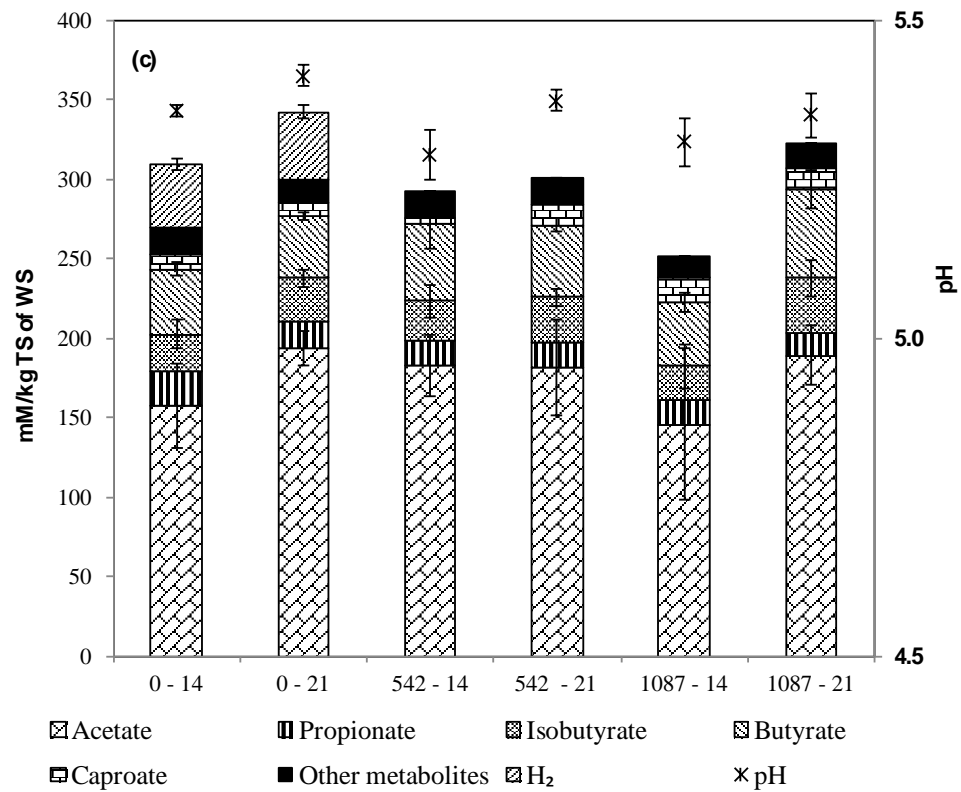


Fig. 2.



503 **Fig. 3.**

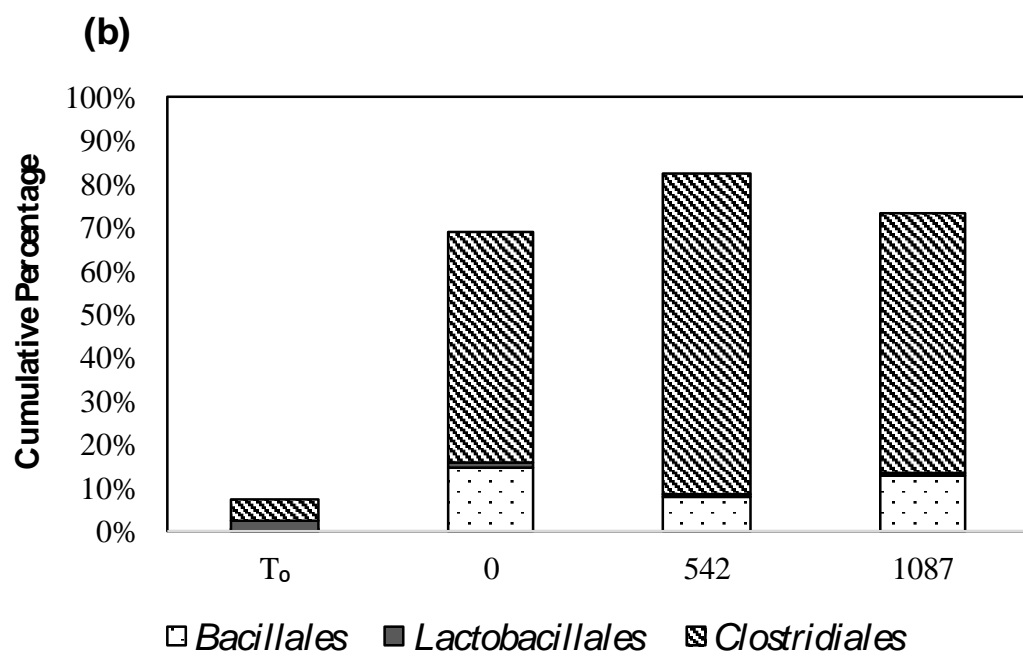
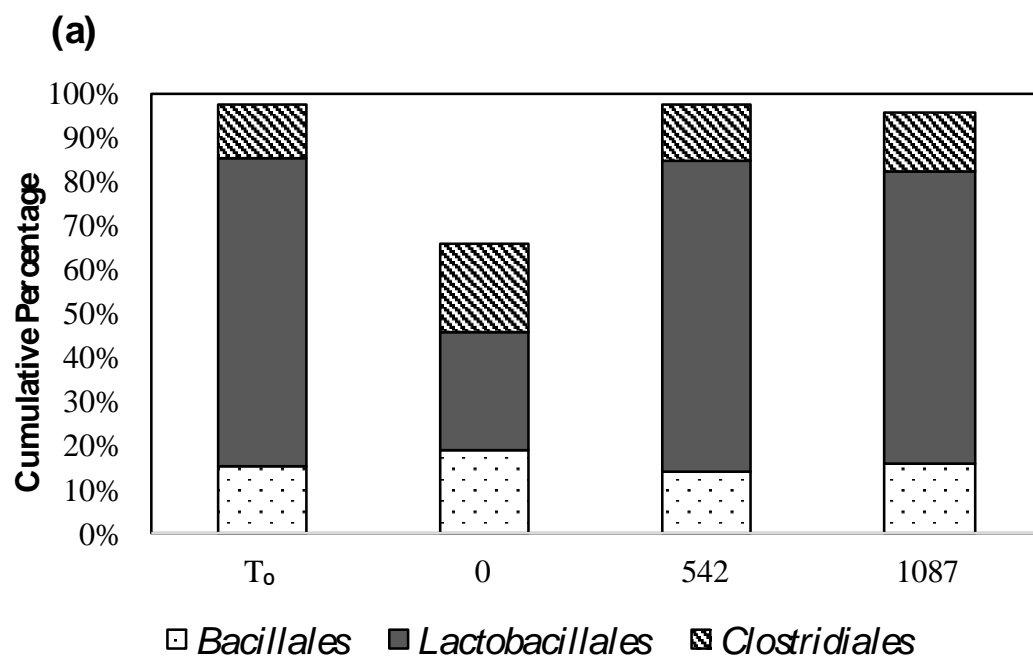




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506 **Fig. 4.**



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