1 **Improved conversion of residual MSW biomass waste to sugars using online process**

2 **monitoring and integrated contamination control**

3 Fernando Climent Barba[ab], Micaela G. Chacón,[b] William R. Reynolds[b], Dhivya J. Puri[c]

4 Richard A. Bourne[b*], A. John Blacker[b]

5 [a]Centre for Doctoral Training in Bioenergy, School of Chemical and Process Engineering,

6 University of Leeds, LS2 9JT, United Kingdom

7 [b]Institute of Process Research and Development, School of Chemistry and School of

8 Chemical and Process Engineering, University of Leeds, LS2 9JT, United Kingdom

9 [c]Fiberight Ltd., Research and Development, Unit 73 Basepoint Enterprise Centre,

10 Southampton SO14 5FE

11 **Correspondence:** Dr Richard A. Bourne, School of Chemical and Process Engineering,

12 University of Leeds, Woodhouse Lane, Leeds, LS2 9JT, UK

13 **E-mail**: R.A.Bourne@leeds.ac.uk

14 **Abstract**

15 Enzymatic saccharification of lignocellulosic biomass to soluble sugars is vulnerable to

16 microbial contamination. Identification of early stage indicators of contamination could

17 allow for more rapid intervention and improved sugar retention. Here we validate the use

18 of dissolved oxygen (DO) and pH as early-stage indicators, and used to evaluate the

19 effectiveness of several antimicrobial agents (sodium azide, benzisothiazolinone,

20 hydrogen peroxide and tetracycline). It was found that benzisothiazolinone (BIT) and

21 sodium azide performed most favourably (8.93 and 9.89 g glucose/L, respectively),

22 controlling contamination for the entire 24 hour time course assessed. We then describe

23 the development of a novel integrated control system with online monitoring of DO/pH

24 and automatic administration of a discrete doses of antimicrobial agent to the reaction

25 once environmental DO dropped below a pre-defined threshold. Further, we show that

26 FTIR can be used for continuous real-time glucose quantification during enzyme

27 hydrolysis as an alternative to traditional offline measurement methods.

28 **Keywords:** Municipal solid waste (MSW); Enzymatic hydrolysis; Microbial growth

29 indicators; Antimicrobial agents; Process monitoring

30 **1 Introduction**

31 Lignocellulosic biomass, which includes materials such as forestry residue, agricultural

32 residue, the organic fraction of municipal solid waste (OFMSW), etc., is an attractive

33 feedstock for biorefineries due to its low cost and widespread availability (Hazar, 2013;

34 Jönsson and Martín, 2016) The hydrolysis of the carbohydrate fraction of lignocellulosic

35 biomass into its constitutive pentose and hexose sugars is of primary interest as they can

36 be further transformed into value-added products (e.g. ethanol, lactic acid or butanol) via

37 chemical conversion or microbial fermentation (Puri et al., 2013; Sluiter et al., 2012). One

38 of the least exploited sources of lignocellulosic biomass is municipal solid waste (MSW),

39 despite it being produced in great quantities worldwide (Jensen et al., 2010). While it

40 varies by region, the lignocellulosic fraction of OFMSW is estimated to be ~30%, of

41 which a significant proportion is composed of plant-derived carbohydrates, making this

42 a viable source of non-food sugar (Hazar, 2013; Meor Hussin et al., 2013).

43 The conversion of lignocellulose to monosaccharides is typically achieved via a two-step

44 process involving an initial pretreatment followed by an enzyme hydrolysis. The role of

the pretreatment step is to upset the natively recalcitrant structure of the biomass and clear away any physical and chemical barriers which render the cellulose less accessible to relevant downstream enzymes (Hendriks and Zeeman, 2009; Jönsson and Martín, 2016; Wahlström and Suurnäkki, 2015). A number of pretreatment regimes are currently in use, and can be broadly classified as: chemical, physical, biological and physiochemical (Bhatia et al., 2020). A large draw back to a number of pretreatment strategies, however, is the generation of degradation products, which are inhibitory to the downstream hydrolysis step. As such, the development of methods to alleviate any pretreatment inhibition issues is an active field of research (Hassan et al., 2018; Jönsson and Martín, 2016). While the secondary hydrolysis step can be carried out either enzymatically or via acid hydrolysis, the former is widely preferred as it offers high selectivity while being a more environmentally benign process (Wahlström and Suurnäkki, 2015). Enzyme hydrolysis is carried out by a cocktail of enzymes working synergistically to deconstruct the lignocellulose. A wealth of work has been carried out to design optimal combinations and ratios of those enzymes present within these cocktails – including but not limited to endoglucanases, cellobiohydrolases, and β-glucosidases, lytic polysaccharide mono-oxygenases (LPMOs), etc. – to maximize hydrolysis for different biomass substrates subjected to different pretreatments (Van Dyk and Pletschke, 2012; Wahlström and Suurnäkki, 2015). However, enzymatic saccharification continues to suffer some techno-economic barriers; including biomass recalcitrance, enzyme costs, end-product inhibition, and low volumetric production compared to food-based sugars (Jönsson and Martín, 2016). Importantly, achieving an economically successful hydrolysis process will require not only improving sugar productivity, but also improving sugar retention.

With the enzymatic hydrolysis of lignocellulosic material resulting in the accumulation of its constitutive monomeric sugars, this step is vulnerable to microbial contamination. Glucose is the primary fuel for microbes, providing the energy required for cell growth and proliferation (Johnston, 1999). As such, any microbial contamination can lead to a significant loss in sugar titres. Preventative measure intended at minimizing the risk of contamination can be carried out prior to enzyme hydrolysis; which could include sterilization of hydrolysis equipment and/or the inclusion of an additional autoclave step to sterilize the lignocellulosic feedstock (Schell et al., 2007; Serate et al., 2015). Both practices possess drawbacks, with the former not always being sufficient to eliminate contamination, and the latter running the risk of generating unwanted inhibitors, as well as increasing the overall time and process cost of a large-scale operation (Hassan et al., 2018). Alternatively, the use of antimicrobial agents during the enzyme hydrolysis can be very effective at controlling contamination. However, a number of considerations need to be made when selecting such an agent, such as: (1) the mode of action of the agent, (2) its short and long term stability in solution, (3) the fate of the sugar rich hydrolysate (i.e. fermentation or chemical conversion), and (4) minimizing the potential of developing antibiotic resistant bacterial strains (Gandla et al., 2018; Islam et al., 1999; Serate et al., 2015). Currently, biological contamination is primarily diagnosed through the detection of organic acids, such as lactic acid, in the sugar rich hydrolysate (Rich et al., 2020). These acids are the end-product of anaerobic fermentation pathways and are therefore indicators of contamination once detected (Serate et al., 2015). Earlier diagnosis could instead by achieved by *in situ* monitoring of upstream environmental indicators of microbial growth and fermentation, specifically, dissolved oxygen (DO) and pH. With oxygen being required for cellular respiration, its consumption increases concomitantly

with microbial population density (Garcia-Ochoa et al., 2010; Riedel et al., 2013). In the absence of constant replenishment, microbial oxygen demand will eventually exceed dissolved oxygen availability, resulting in its depletion. In the absence of oxygen, a number of microbial species will metabolically switch to anaerobic fermentation for energy synthesis, the product(s) of which are short chain alcohols and acids (Ward, 2014). Consequently, the accumulation of these products results in a drop in environmental pH. Thus, an observed drop in environmental DO followed by a drop in pH is a classic indication of microbial growth and fermentation (Deepak et al., 2008; Famelart et al., 1987).

Generally, enzyme hydrolyses are poorly monitored over the course of the reaction, with sugar and acid quantification being carried out intermittently by offline methods such as HPLC and enzyme assay, while DO and pH are rarely tracked (Gandla et al., 2018). Herein, we validate the use of DO and pH as metrics for the diagnosis of early stage microbial contamination during enzymatic hydrolysis of MSW pulp, and use them to evaluate the effectiveness of a number of antimicrobial agents (sodium azide, benzisothiazolinone, hydrogen peroxide, and tetracycline). We then demonstrate the use of an integrated control system with continuous process monitoring of DO and pH and a custom-made operating system designed to automatically issue a defined dose of a given antimicrobial agent once environmental DO has dropped below a pre-set threshold. Further, we show here the first use of *in situ* FTIR for real-time sugar quantification during lignocellulosic biomass hydrolysis as an alternative to the more commonly used offline techniques, such as HPLC and enzyme assays. This work lays the foundation for entirely online monitoring of enzymatic hydrolysis reactions, the application of which could improve both sugar production and sugar retention.

2 Materials and Methods

Cellic® CTec3 was kindly donated by Novozymes (Copenhagen, Denmark) and Fermasure® was purchased from Dupont, Ltd. (London, UK). All other chemicals and reagents were purchased from Sigma Aldrich (Dorset, UK) or Fisher Scientific (Loughborough, UK) unless stated otherwise.

MSW pulp was provided by Fiberight Ltd. from its pilot plant in Lawrenceville (Virginia, USA). Plastics and metals were removed and the lignocellulosic fraction (consisting of mostly paper and card) was pulped by a series of hydrothermal processes. The resulting fibrous material was supplied at a dry matter of 50-55%, parameter determined by the "oven-drying method". The lignocellulose composition was determined to be 55% glucan, 12% xylan, 6% araban/galactan/mannan, 24% lignin, 3% ash (Puri et al., 2013).

2.1 Enzymatic hydrolysis of MSW pulp

Enzymatic hydrolysis was carried out in a 1 L jacketed-vessel (Scientific UK) for 24 hours at 50 °C, agitated with a 4 pitched-blade impeller (Caframo Limited, Ontario, Canada) at 700 rpm. Hydrolysis reactions were carried out using either 6% or 7% total solids (TS, %)of MSW pulp and 2% (w/w dry substrate)) CTec3 enzyme cocktail in a reaction medium of water, totalling a working mass of 800 g. Slurry pH was adjusted to 5.25 using 6% phosphoric acid and was then incubated at 50 °C for 30 minutes prior to the addition of enzyme. 0.1% (w/w dry substrate) of either NaN_3 (sodium azide), BIT (benzisothiazolinone), H_2O_2 (hydrogen peroxide) or , tetracycline were added to the reaction slurry immediately prior to the addition of CTec3 at the beginning of the reaction, or once DO reached 0 mg/L (typically at hour 6). For those reactions, antimicrobial agent

138 was added once the DO had reached 0 mg/L, slurry pH was adjusted using 10 % sodium

139 hydroxideif it dropped below 4.75 for the first 8 hours.

140 Samples from the hydrolysis slurry were withdrawn from a well-mixed region close to

141 the impeller and centrifuged at 4000 rpm for 15 minutes. The hydrolysate was then passed

142 through a 0.45 μm filter and stored frozen at -20 °C until analysis.

143 *2.2 Integrated control system*

144 To prevent the growth of microorganisms in the sugar-rich reaction mass, an integrated

145 control system was designed with three main sub-systems: (1) process monitoring, (2)

146 operating system, (3) automatic dosing system. A schematic of this set up is shown in

147 Fig. S1. During enzymatic hydrolysis, the pH and DO were measured with the

148 corresponding sensors: InLaB® probes (previously calibrated with vendor standards)

149 installed in the SevenExcellence™ multi-parameter kit. Continuous recordings (1 minute

150 frequency) were automatically transferred to, Labx direct pH 3.3 (Mettler Toledo, USA)

151 monitoring software, generating *.txt files*. A bespoke operating system, named "*glucose*

152 *bioreactor model*", was programmed using LabVIEW (National Instruments, UK)

153 (Blacker et al., 2019)This incorporates on-line data and commands the automatic dosing

154 of anti-microbial agents by a syringe-pump unit (model 11, Harvard apparatus UK)

155 according to pre-defined settings (SI, Fig.S2) whereby a DO gradient threshold (ΔDO) is

156 set as an "alarm" for triggering the sterilising product: $\Delta DO < - 0.028$ mg L^{-1} s^{-1}. The

157 algorithm calculates the DO gradient according to the equation shown below. A full

158 description of the *glucose bioreactor model* can be found in the (SI, Fig.S2).

159 $$\Delta D = \frac{DO_F - DO_{f'}}{f} \qquad\qquad (Eq.1)$$

160 Where: DO_F is the last monitored DO reading (mg/L), DO_f the value prior DO_F (mg/L)

161 and f is the frequency of monitoring (in s, e.g. 10 s).

162 *2.3 Quantification of microbial population*

163 Samples of slurry were taken at discrete time points during a hydrolysis reaction and

164 plated at several dilutions on Luria Broth agar plates (10 g/L tryptone, 10 g/L NaCl, 5 g/L

165 yeast extract, 15 g/L agar). Plated samples were incubated at 50 °C for 16 hours.

166 Following incubation, colony forming units (CFUs) were counted and dilution corrected.

167 *2.4 Quantification of products*

168 Monomeric sugar and acid concentrations were quantified by HPLC. Monosaccharides

169 (D-glucose and D-xylose) and organic acids (L-lactic acid and acetic acid) were analysed

170 simultaneously by HPLC fitted with an Ultimate^{TM} Dionex 3000, UK column. A 10 μL

171 sample was injected and separated by a Supelcogel™ C-610H (6% Crosslinked) column

172 with a deashing guard column, operating at 30 °C with 0.1 wt% phosphoric acid at a flow

173 rate of 0.5 ml.min^{-1} as mobile phase. Monosaccharides and organic acids were detected

174 respectively by a Shodex RI-101 refractive index and a diode array detector (Thermo

175 Scientific, UK). High-purity analytical standards used to calibrate each product to

176 determine linear response concentrations. All samples were run in duplicate and

177 chromatograms were processed by Chromoleon software$^{®}$.

178 On-line sugar analysis was performed by *in-situ* FTIR, monitoring sugar formation using

179 a MB3000 FTIR instrument (ABB, Switzerland). As glucose has an absorption peak at a

180 wavelength of 1035 cm^{-1}, a calibration curve was created by plotting absorbance at

181 1035cm^{-1} relative to 0-100 g/L glucose concentration (SI, Fig.S3). After calibration, the

182 probe was fitted in a 2-L stirred tank reactor to monitor an enzymatic saccharification,

183 with absorbance (at 1035 cm^{-1}) readings taken every 120s by averaging 3 scans. FTIR

184 values were translated into g/L by the above-mentioned calibration curve in order

185 compare them with HPLC ones of given samples.

186 **3 Results and Discussion**

187 *3.1 Determining microbial contamination via pH/DO monitoring*

188 Both pH and DO are closely monitored and controlled during microbial fermentations in

189 order to optimize growth and productivity (Famelart et al., 1987; Jones et al., 1992;

190 Mohd-Zaki et al., 2016). Despite this, they are rarely used as metrics during enzyme

191 hydrolysis to evaluate the presence of microbial contamination. To validate that DO and

192 pH can be used as diagnostic tools for the detection of microbial contamination during

193 enzymatic hydrolysis of lignocellulose, a saccharification control reaction was run using

194 6% MSW pulp and 2% C-Tec3 enzyme cocktail. No pre-hydrolysis pulp sterilization

195 measures were taken nor any antimicrobial agents used, ensuring that the untreated pulp

196 was microbially compromised. This was confirmed as samples taken from said pulp

197 showed a mixed population of bacteria (SI, Fig. S4). During this reaction, it was found

198 that DO fluctuated for approximately the first 2 hours as the rheology of the slurry

199 changed due to the rapid initial activity of cellulases/endoglucanases, resulting in the

200 release of air from within the porous MSW matrix. During the first several hours, the

201 microbial population proliferated as DO and glucose were in abundance – the latter being

202 continuously released from the lignocellulose substrate (Fig. 1a-c). However, as there was

203 no continuous oxygen supplementation into the reaction mixture, eventually population

204 density reached a threshold whereby microbial oxygen demand exceeded its availability

205 (Fig. 2c). This resulted in a steep drop in DO by hour 5.5, with 0 mg/L being reached by

hour 6 (Fig. 1a). At this point, the environmental pH began to drop as those species capable of fermentation began to produce short chain acids. An analysis of reaction products over the 24 hour time course showed that lactic acid began to accumulate between hours 4-8 (Fig. 1b) and continued to do so over the course of the reaction, culminating in a final titre of 140 mg/L at hour 24. The onset of lactic acid production correlates well with the observed drop in environmental pH of the reaction, between hours 6-8. Lactic acid is a common product of microbial fermentation induced by anaerobic conditions (Othman et al., 2017), and its presence is widely considered a symptom of contamination (Serate et al., 2015). Unlike lactic acid, acetic acid was detected within two hours of the start of the reaction and continued to increase slowly over the 24-hour time course to a final concentration of 60 mg/L (Fig. 1b). While acetic acid can also be a fermentation product from a number of microbial species (Raspor and Goranovič, 2008), it is also released upon the hydrolysis of acetyl groups from hemicellulose during saccharification (Jönsson and Martín, 2016; Serate et al., 2015)). Thus, unlike lactic acid, it is not a definitive indicator of microbial contamination. Final glucose titres did not change between hours 8-24 (Fig. 1b) which would suggest that glucose production and glucose utilization – either through microbial fermentation to lactic acid or biomass accumulation – are in equilibrium.

Having confirmed that a drop in environmental DO and pH during the enzymatic hydrolysis of lignocellulose is correlated with the early growth phase of a microbial population, we used this criterion to time the application of an antimicrobial dose into the reaction mixture in order to halt microbial proliferation and improve glucose yield (Fig. 2a-c). The hydrolysis was carried out under the same conditions as described above, with the exception that once DO reached 0 mg/L, just before hour 6, 0.1% (w/w dry substrate)

230   BIT, a bactericidal antimicrobial, was manually injected into the slurry. Nearly

231   immediately, the DO rose to 5 mg/L, and an analysis of CFUs present within the slurry

232   at hour 8 showed a significant reduction in population density, with a further drop by hour

233   24 (Fig. 2c). It has previously been found that during microbial death phase, that dissolved

234   oxygen increases as its overall consumption is reduced – explaining this observed pattern

235   (Riedel et al., 2013). Further, it was found that pH, which had begun to drop slightly

236   following the early state of DO exhaustion, also stabilized after the addition of the

237   antimicrobial dose (Fig. 2a). While this would suggest fermentation had begun prior to

238   the BIT addition, no lactic acid was detected throughout the time course. Conversely,

239   acetic acid production was observed from hour 2 onwards. Further, unlike the control

240   reaction which had no antimicrobial supplementation, glucose titres continued to rise

241   between 8-24 hours, resulting in a final titre of 9.5 g/L for the reaction in which BIT was

242   added. While presumably a small amount glucose will have been converted to microbial

243   biomass prior to the BIT addition, this still represents a 40% improvement in glucose

244   production compared to the no antimicrobial control.

245   Ultimately, monitoring DO and pH over the course of an enzymatic hydrolysis of

246   lignocellulose represents a strategy that is easy to implement and could allow for earlier

247   detection of microbial contamination compared to the more commonly used downstream

248   indicator, such as the accumulation of short chain acids (Serate et al., 2015). Potentially

249   enabling faster intervention and therefore maximization of sugar production. This is

250   especially useful for large industrial-scale monomeric sugar production where pre-

251   sterilization of pulp via autoclave may be unrealistic due to the high capital and operating

252   costs, while pasteurization of hydrolysis equipment is not always effective at eliminating

253   contamination (Rich et al., 2020; Serate et al., 2015). Further, the continuous monitoring

254   of DO and pH during the hydrolysis of lignocellulose can have the additional benefit of

255   ensuring consistently optimal conditions for the protein mediated saccharification. Those

256   enzymes present within the majority of commercial cocktails work optimally within a

257   specific pH range, while the recent discovery of lytic polysaccharide monooxygenase

258   enzymes and their utilization of molecular oxygen as a co-substrate highlights the need

259   to closely control both these factors (Du et al., 2012; Fenila and Shastri, 2016; Gusakov

260   et al., 2017).

261   *3.2 Tackling microbial contamination by several antimicrobial agents according to*

262   *pH/DO metrics*

263   With MSW pulp typically containing a mixed microbial population of bacteria, yeast and

264   fungal species (Hassen et al., 2001) the selection of an antimicrobial agent with broad

265   spectrum activity becomes highly important. Following on from the results with BIT in

266   Section 3.1, we sought to demonstrate the application of DO and pH as metrics to evaluate

267   the efficacy of a number of other commonly used antimicrobial agents to control the

268   proliferation of a mixed microbial population during the enzymatic hydrolysis of

269   unsterilized MSW pulp. To test this, MSW saccharification time courses were run as

270   described in section 3.1, where once DO reached 0 mg/L, a 0.1% (w/w dry substrate) dose

271   of either: sodium azide (NaN_3), hydrogen peroxide (H_2O_2) or tetracycline was manually

272   injected into the slurry, and pH, DO, monomeric sugars, and organic acids were

273   monitored for 24 hours (Fig. 3a-f). A comparison of each time course demonstrated that

274   a drop in the measured values for both DO and pH were good indicators of infection and

275   correlated well with microbial viability as determined by the number of CFU/ml (Fig. 5).

276   Of the four antimicrobials tested, the most effective were BIT (Fig. 2a-c) and NaN_3 (Fig.

277   3a-c), while H_2O_2 and tetracycline were found to be insufficient at managing microbial

278   population over a 24 hour hydrolysis (Fig. 3c-f). This trend was additionally observed in

279   experiments where the antimicrobial dose was added at the onset of hydrolysis (Fig. S7a-

280   f).

281   The mode of action of NaN_3 is to inhibit the respiratory chain of gram-negative bacteria,

282   acting as a bacteriostat (Cabrol et al., 2017; Russo et al., 2008). Fig. 5 shows that the

283   microbial population density remained relatively unchanged after the addition of NaN_3.

284   This would suggest that the majority of the microbial population contaminating MSW

285   pulp are gram negative bacterial species. This is interesting as the presence of lactic acid

286   contamination is often attributed to the order of gram positive lactic acid bacteria (LAB)

287   (Othman et al., 2017), despite a number of gram negative bacteria, as well as fungal

288   species, also being able to produce lactic acid (Förster and Gescher, 2014; Lin et al.,

289   2018). It was also observed that the hydrolysis supplemented with NaN_3 resulted in the

290   highest glucose recovery, with 10 g/L at 24 hours, which is approximately a 30%

291   improvement over the no antimicrobial control (Figs. 2b and 4b). Whilst NaN_3 is a stable

292   under the reaction conditions, its recalcitrance and toxicity prevent downstream use of

293   the sugar in fermentation processes, so for these applications BIT is preferable.

294   Compared to NaN_3, BIT supplementation resulted in a drastic drop in the total microbial

295   population over the 24 hour time course (Fig. 2a-b). This is not surprising as BIT is

296   described as broad spectrum antimicrobial, possessing bactericidal and fungicidal activity

297   (Silva et al., 2020; Williams, 2006). Interestingly, while no lactic acid was detected in the

298   hydrolysis reaction that had BIT supplementation, glucose titres were not as high as those

299   where NaN_3 was used. Given the significant impact BIT had on microbial population

300 density within the slurry, as well as the absence of any fermentation products, it might

301 suggest that the presence of BIT is hindering glucose formation by other means – perhaps

302 by inhibiting the enzyme activity of the cellulolytic cocktail used for hydrolysis. To test

303 this, we reduced the concentration of BIT supplementation to 0.05% (w/w dry substrate),

304 and found that glucose yield was 10% higher than those hydrolyses supplemented with

305 0.1% (w/w dry substrate) BIT (SI, Fig. S5c). At the lower BIT concentration, final glucose

306 titres after 24 hours were similar to those obtained when NaN_3 was used, with no

307 detectable presence of fermentation products. Persistence of BIT in hydrolysate may

308 posse several issues for further utilisation of sugars, this can be mitigate by neutralisation

309 and distillation steps during downstream processing which decompose BIT into less

310 hazardous molecules (Silva et al., 2020).

311 In contrast to NaN_3 and BIT, neither tetracycline not hydrogen peroxide was found to be

312 as long lasting in managing the microbial contamination during OFMSW hydrolysis.

313 Hydrogen peroxide is a commonly used disinfectant that works via the oxidation of

314 essential biomolecules, causing damage to proteins, nucleic acids, and lipids (McDonnell,

315 2014). It has been found to be effective against both gram-positive and gram-negative

316 bacteria, fungi, and yeast (McDonnell, 2014). The manual injection of 0.1% (w/w dry

317 substrate) hydrogen peroxide at hour 6 of a hydrolysis reaction resulted in a concomitant

318 rise in dissolved oxygen, however it dropped again to 0 mg/L 2.5 hours later, and

319 remained at 0 mg/L for the remainder of the hydrolysis (Fig. 3c-d). Following the drop in

320 DO at 8.5 hours, the environmental pH also dropped significantly, eventually reaching

321 4.5 by the end of the time course. Between hours 8 to 24 there was a significant

322 accumulation of both lactic acid and acetic acid, explaining this observed drop in

323 environmental pH (Fig. 3c). While the accumulation of acetic acid can be partially

explained by the hydrolysis of hemicellulose, the sharp rise in its concentration at hour 24 could suggest that some may have been produced microbially as a product of fermentation. These results mirrored well in the analysis of microbial population density over the course of the hydrolysis. An analysis of the number of CFUs showed a drop population density at hour 8, after the addition of hydrogen peroxide, followed by a significant resurgence by hour 24 (Fig. 5). This would suggest that while the addition of hydrogen peroxide had an initial antimicrobial effect, that it was short lived. This agrees with work carried out by Alt et al., 1999, who found that concentrations below 3% hydrogen peroxide were not effective at reducing microbial growth. Hydrogen peroxide is sensitive to self-decomposition, especially at elevated temperatures (Serra-Maia et al., 2018)or by the activity of catalase enzymes which are commonly produced by microbial species. This would suggest that a concentration of 0.1% (w/w dry substrate) of hydrogen peroxide decomposes far too quickly to have long lasting antimicrobial effects. However, we found that while supplementation of concentrations above 0.4% was successful in abolishing microbial contamination, it also dramatically reduced the rate of enzymatic saccharification (SI, Fig. S6), suggesting that an excess of hydrogen peroxide inhibits cellulase activity. However, with the recent discovery that hydrogen peroxide can act as a co-substrate for LPMO enzymes (Müller et al., 2018), it may be the case that a fed batch system where hydrogen peroxide is continuously supplied at a low concentration may have the dual benefit of improving saccharification (via improved LPMO activity) and controlling microbial contamination.

Tetracycline is a well-established bacteriostat that exhibits activity against a wide range of microorganisms including gram-positive and gram-negative bacteria, mycoplasmas, rickettsiae, chlamydiae, as well as some eukaryotic parasites (Van Bambeke et al., 2017).

348 It was found that the manual addition of 0.1% (w/w dry substrate) tetracycline once DO

349 dropped to 0 mg/L resulted in the rise and stabilization of DO and environmental pH for

350 approximately 7 hours before DO dropped once again to 0 mg/L at hour 14 (Fig. 3e). An

351 analysis of the number of CFUs at hour 8 showed a significant drop in microbial

352 population density; however, a considerable resurgence in population had occurred by

353 hour 24 (Fig. 5). Despite this, only a small amount of lactic acid (30 mg/L) was detected

354 at this final time point – suggesting the population was skewed towards non-fermenting

355 species. The apparent loss of effectiveness of tetracycline after 7 hours could be attributed

356 to several factors. Tetracycline has been found to be sensitive to both light and high

357 temperatures (López-Peñalver et al., 2010). While the former was not controlled for in

358 this work, the latter is a necessity for effective cellulase enzyme activity (with the reaction

359 temperature being maintained at 50 ºC). The necessity for high temperatures during

360 lignocellulosic hydrolysis suggests that this antibiotic is not ideal for these purposes.

361 Furthermore, the use of antibiotics, such as tetracyclines, for the control of microbial

362 populations for non-medical purposes is controversial in the current climate of antibiotics

363 overuse and resistance.

364 Ultimately, the choice of antimicrobial agent will also depend on a number of process

365 specific factors, including the degree and type of microbial contamination of the chosen

366 feedstock, and the eventual fate of the sugar rich hydrolysate (i.e. feedstock for microbial

367 fermentation or chemical conversion).

368 *3.3 Integrated control system with online pH/DO monitoring and automated*

369 *antimicrobial dosing*.

370　With the success of using DO and pH for the early determination of microbial infection,

371　an automated antimicrobial dosing system was developed that could be employed in a

372　production process in order to protect sugar yield and provide a more consistent quality.

373　This automation also has the advantage that experiments can be run overnight

374　unsupervised. The integrated control system developed here involves measuring and

375　logging DO and pH data and using a bespoke algorithm to detect a consistent fall of both

376　metrics. Since DO was found to be an earlier and more useful measure of infection, the

377　algorithm was designed to trigger a pump to deliver a specific dose of anti-microbial agent

378　when the gradient on the DO trace exceeds - 0.028 mg L^{-1} s^{-1} (SI, FigS2). A stand-alone

379　pH measurement was recorded simultaneously. The system was tested without

380　antimicrobial (Fig. 5a), and with NaN_3 alone (Fig. 5b). While NaN_3 was used here to

381　demonstrate the automatic dosing system, the agent can easily be substituted for another

382　(ex. BIT). At the start of the process, the viscosity of the system is high, however after

383　1-2 hours a rheological change was observed (viscosity-break shown by the hatched

384　boxes), and causing fluctuations in the DO reading. Upon detecting a sustained fall in DO

385　at around 5.6 hours, a 10-ml dose of NaN_3 (0.01%, w/w dry substrate) was automatically

386　administered which, over the next hour, resulted in its recovery to pre-infection saturation

387　levels (Fig. 5b). In the control run (Fig 6a), the DO gradient was also plotted during the

388　course of hydrolysis for comparison purposes, even though no automated dosing

389　occurred. A final quantification of glucose at hour 20 revealed that the hydrolysis with no

390　antimicrobial dosing resulted in 10.8 g/L, while the hydrolysis with automatic NaN_3

391　dosing accumulated 17.2 g/L. Automated detection and dosing can improve the process

392　economics, maximise sugar production and improve product consistency.

393　*3.4 Continuous glucose monitoring during enzymatic hydrolysis of MSW pulp*

While monitoring of DO and pH provides invaluable information about the state of microbial contamination during the enzymatic hydrolysis of a lignocellulosic feedstock, the purpose of this knowledge is ultimately to use it to maximize sugar yields from the reaction. As such, it is equally valuable to monitor sugar concentration throughout the hydrolysis process (Landari et al., 2018). Currently, these measurements are predominantly carried out offline, using chromatography techniques, such as HPLC, or glucose monitors. While the former technique is time consuming, it benefits from being able to resolve complex sugar mixtures within the slurry, while the latter technique is quicker but specific for only one sugar in the mixture (Leopold et al., 2011). Both techniques tend to be only carried out intermittently throughout the course of the reaction, ultimately resulting in delayed information about total sugar concentration.

While *in situ* sugar monitoring using Fournier-Transform Infrared Spectroscopy (FTIR) has been performed previously for sugar quantification in fruit juices and other sugar based products (Landari et al., 2018; Wang et al., 2010), it appears that FTIR has not previously been used for real-time glucose measurement over the course of a lignocellulosic hydrolysis (Sills and Gossett, 2012; Tucker et al., 2001). This is unsurprising given the nature of the slurries involved, which are complex and dynamically changing heterogeneous mixture. In order to achieve a real-time *in-situ* monitoring system for glucose during the enzymatic hydrolysis of MSW pulp, we used FTIR to continuously measure changes in adsorption. A wavenumber of 1035 cm^{-1} was chosen for glucose quantification as it has previously been shown to possess the most linear correlation between absorption and glucose concentration (Nybacka, 2016). An enzymatic hydrolysis was carried out using 7% TS and 5% C-Tec3 (w/w dry substrate), without antimicrobial supplementation. The absorbance readings were converted into

418   glucose concentrations based on a previously created standard curve, (SI, Fig.S3) and

419   compared to periodic samples taken and analysed by HPLC (Fig. 6). Both quantification

420   strategies are tightly correlated, though the glucose concentrations measured by FTIR are

421   consistently higher than those values obtained by HLPC. This is potentially a result of the

422   FTIR method also detecting xylose, cellobiose and other soluble oligomeric sugars.

423   Additionally, intermittent HPLC analysis of glucose plus xylose were also included as

424   an upper threshold. Online FTIR values fit considerably well within those limits, HPLC

425   glucose (below) and HPLC glucose plus xylose (above). This data validates the use of

426   FTIR as an accurate and significantly more rapid method for monomeric sugar

427   determination and has potential to be adopted as a control method within a production

428   process (Blacker et al., 2019).

429   **4. Conclusions**

430   The present study shows that antimicrobial agents are effective in raising MSW sugar

431   hydrolysate titres. BIT and NaN_3 were identified as efficacious antimicrobial agents; the

432   former may be preferred if the sugar is used for fermentations, whilst the latter may be

433   more useful for longer term storage and speciality chemical applications. DO is shown to

434   be a good microbial growth indicator and has been integrated into an operating system

435   for automated mitigation of biological infection in sugar solutions. On-line measurements

436   (pH/DO and FTIR-based glucose monitoring) are useful tools to monitor and control the

437   saccharification process of lignocellulosic feedstocks.

References

Alt, E., Leipold, F., Milatovic, D., Lehmann, G., Heinz, S., Schömig, A., 1999. Hydrogen peroxide for prevention of bacterial growth on polymer biomaterials. Ann. Thorac. Surg. 68, 2123–2128.

Bhatia, S.K., Jagtap, S.S., Bedekar, A.A., Bhatia, R.K., Patel, A.K., Pant, D., Rajesh Banu, J., Rao, C. V., Kim, Y.G., Yang, Y.H., 2020. Recent developments in pretreatment technologies on lignocellulosic biomass: Effect of key parameters, technological improvements, and challenges. Bioresour. Technol. 300, 122724.

Blacker, A.., Bourne, R.., Reynolds, W.., 2019. MSW Process Control Systems. WO2019220092.

Cabrol, L., Quéméneur, M., Misson, B., 2017. Inhibitory effects of sodium azide on microbial growth in experimental resuspension of marine sediment. J. Microbiol. Methods 133, 62–65.

Deepak, V., Kalishwaralal, K., Ramkumarpandian, S., Babu, S.V., Senthilkumar, S.R., Sangiliyandi, G., 2008. Optimization of media composition for Nattokinase production by Bacillus subtilis using response surface methodology. Bioresour. Technol. 99, 8170–8174.

Du, R., Su, R., Li, X., Tantai, X., Liu, Z., Yang, J., Qi, W., He, Z., 2012. Controlled

463   adsorption of cellulase onto pretreated corncob by pH adjustment. Cellulose 19,

464   371–380.

465   Famelart, M.H., Kobilinsky, A., Bouillanne, C., Desmazeaud, M.J., 1987. Influence of

466   temperature, pH and dissolved oxygen on growth of Brevibacterium linens in a

467   fermentor. Appl. Microbiol. Biotechnol. 25, 442–448.

468   Fenila, F., Shastri, Y., 2016. Optimal control of enzymatic hydrolysis of lignocellulosic

469   biomass. Resour. Technol. 2, S96–S104.

470   Förster, A.H., Gescher, J., 2014. Metabolic engineering of Escherichia coli for

471   production of mixed-acid fermentation end products. Front. Bioeng. Biotechnol. 2,

472   16.

473   Gandla, M.L., Martín, C., Jönsson, L.J., 2018. Analytical Enzymatic Saccharification of

474   Lignocellulosic Biomass for Conversion to Biofuels and Bio-Based Chemicals.

475   Energies 11, 2936.

476   Garcia-Ochoa, F., Gomez, E., Santos, V.E., Merchuk, J.C., 2010. Oxygen uptake rate in

477   microbial processes: An overview. Biochem. Eng. J. 49, 289–307.

478   Gusakov, A. V., Bulakhov, A.G., Demin, I.N., Sinitsyn, A.P., 2017. Monitoring of

479   reactions catalyzed by lytic polysaccharide monooxygenases using highly-sensitive

480   fluorimetric assay of the oxygen consumption rate. Carbohydr. Res. 452, 156–161.

481   Hassan, S.S., Williams, G.A., Jaiswal, A.K., 2018. Emerging technologies for the

482   pretreatment of lignocellulosic biomass. Bioresour. Technol. 262, 310–318.

483   Hassen, A., Belguith, K., Jedidi, N., Cherif, A., Cherif, M., Boudabous, A., 2001.

484   Microbial characterization during composting of municipal solid waste. Bioresour.

485   Technol. 80, 217–225.

486   Hazar, Y., 2013. Sources for Lignocellulosic Raw Materials for the Production of

487   Ethanol, in: Lignocellulose Conversion: Enzymatic and Microbial Tools for

488   Bioethanol Production. pp. 21–38.

489   Hendriks, A.T.W.M., Zeeman, G., 2009. Pretreatments to enhance the digestibility of

490   lignocellulosic biomass. Bioresour. Technol. 100, 10–18.

491   Islam, M., Toledo, R., Hamdy, M.K., 1999. Stability of virginiamycin and penicillin

492   during alcohol fermentation. Biomass and Bioenergy 17, 369–376.

493   Jensen, J.W., Felby, C., Jørgensen, H., Rønsch, G.Ø., Nørholm, N.D., 2010. Enzymatic

494   processing of municipal solid waste. Waste Manag. 30, 2497–2503.

495   Johnston, M., 1999. Feasting, fasting and fermenting: Glucose sensing in yeast and

496   other cells. Trends Genet. 15, 29–33.

497   Jones, K.O., Williams, D., Montgomery, P.A., Phipps, D., 1992. Dissolved oxygen

498   control in a fermentation of Saccharomyces cerevisae using an automatic tuning

499   PID controller, in: IFAC Symposia Series. Publ by Pergamon Press Inc, pp. 247–

500   250.

501   Jönsson, L.., Martín, C., 2016. Pretreatment of lignocellulose: Formation of inhibitory

502   by-products and strategies for minimizing their effects. Bioresour. Technol. 199,

503   103–112.

504   Landari, H., Roudjane, M., Messaddeq, Y., Miled, A., 2018. Pseudo-continuous flow

505   FTIR system for glucose, fructose and sucrose identification in Mid-IR range.

506   Micromachines 9.

507   Leopold, L.F., Leopold, N., Diehl, H.A., Socaciu, C., 2011. Quantification of

508   carbohydrates in fruit juices using FTIR spectroscopy and multivariate analysis.

509   Spectroscopy 26, 93–104. https://doi.org/10.3233/SPE-2011-0529

510   Lin, Y.C., Cornell, W.C., Jo, J., Price-Whelan, A., Dietrich, L.E.P., 2018. The

511   pseudomonas aeruginosa complement of lactate dehydrogenases enables use of d -

512   and l -lactate and metabolic cross-feeding. MBio 9.

513   López-Peñalver, J.J., Sánchez-Polo, M., Gómez-Pacheco, C. V., Rivera-Utrilla, J.,

514   2010. Photodegradation of tetracyclines in aqueous solution by using UV and

515   UV/H2O2 oxidation processes. J. Chem. Technol. Biotechnol. 85, 1325–1333.

516   McDonnell, G., 2014. The Use of Hydrogen Peroxide for Disinfection and Sterilization

517   Applications, in: PATAI'S Chemistry of Functional Groups. John Wiley & Sons,

518   Ltd, Chichester, UK, pp. 1–34.

519   Meor Hussin, A.S., Collins, S.R.A., Merali, Z., Parker, M.L., Elliston, A., Wellner, N.,

520   Waldron, K.W., 2013. Characterisation of lignocellulosic sugars from municipal

521   solid waste residue. Biomass and Bioenergy 51, 17–25.

522   Mohd-Zaki, Z., Bastidas-Oyanedel, J., Lu, Y., Hoelzle, R., Pratt, S., Slater, F., Batstone,

523   D., 2016. Influence of pH Regulation Mode in Glucose Fermentation on Product

524   Selection and Process Stability. Microorganisms 4, 2.

525   Müller, G., Chylenski, P., Bissaro, B., Eijsink, V.G.H., Horn, S.J., 2018. The impact of

526   hydrogen peroxide supply on LPMO activity and overall saccharification

527   efficiency of a commercial cellulase cocktail. Biotechnol. Biofuels 11, 209.

528   Novozymes, 2012. Novozymes Cellic® CTec3 - Secure your plant's lowest total cost.

529   Novozymes 1–6.

530   Nybacka, L., 2016. FTIR spectroscopy of glucose. Uppsala Universitet.

531   Othman, M., Ariff, A.B., Rios-Solis, L., Halim, M., 2017. Extractive fermentation of
532   lactic acid in lactic acid bacteria cultivation: A review. Front. Microbiol.

533   Puri, D.J., Heaven, S., Banks, C.., 2013. Improving the performance of enzymes in
534   hydrolysis of high solids paper pulp derived from MSW. Biotechnol. Biofuels 6,
535   107.

536   Raspor, P., Goranovič, D., 2008. Biotechnological applications of acetic acid bacteria.
537   Crit. Rev. Biotechnol. 28, 101–124.

538   Rich, J.O., Anderson, A.M., Leathers, T.D., Bischoff, K.M., Liu, S., Skory, C.D., 2020.
539   Microbial contamination of commercial corn-based fuel ethanol fermentations.
540   Bioresour. Technol. Reports 11, 100433.

541   Riedel, T.E., Berelson, W.M., Nealson, K.H., Finkel, S.E., 2013. Oxygen consumption
542   rates of bacteria under nutrient-limited conditions. Appl. Environ. Microbiol. 79,
543   4921–4931.

544   Russo, I., Del Mese, P., Viretto, M., Doronzo, G., Mattiello, L., Trovati, M., Anfossi,
545   G., 2008. Sodium azide, a bacteriostatic preservative contained in commercially
546   available laboratory reagents, influences the responses of human platelets via the
547   cGMP/PKG/VASP pathway. Clin. Biochem. 41, 343–349.

548   Schell, D.J., Dowe, N., Ibsen, K.N., Riley, C.J., Ruth, M.F., Lumpkin, R.E., 2007.
549   Contaminant occurrence, identification and control in a pilot-scale corn fiber to
550   ethanol conversion process. Bioresour. Technol. 98, 2942–2948.

551   https://doi.org/10.1016/j.biortech.2006.10.002

552 Serate, J., Xie, D., Pohlmann, E., Donald, C., Shabani, M., Hinchman, L., Higbee, A.,

553   Mcgee, M., La Reau, A., Klinger, G.., Li, S., Myers, C.., Boone, C., Bates, D..,

554   Cavalier, D., Eilert, D., Oates, L.., Sanford, G., Sato, T.., Dale, B., Landick, R.,

555   Piotrowski, J., Ong, R.., Zhang, Y., 2015. Controlling microbial contamination

556   during hydrolysis of AFEX-pretreated corn stover and switchgrass: effects on

557   hydrolysate composition, microbial response and fermentation. Biotechnol.

558   Biofuels 8, 180.

559 Serra-Maia, R., Bellier, M., Chastka, S., Tranhuu, K., Subowo, A., Rimstidt, J.D., Usov,

560   P.M., Morris, A.J., Michel, F.M., 2018. Mechanism and Kinetics of Hydrogen

561   Peroxide Decomposition on Platinum Nanocatalysts. ACS Appl. Mater. Interfaces

562   10, 21224–21234.

563 Sills, D.L., Gossett, J.M., 2012. Using FTIR to predict saccharification from enzymatic

564   hydrolysis of alkali-pretreated biomasses. Biotechnol. Bioeng. 109, 353–362.

565 Silva, V., Silva, C., Soares, P., Garrido, E.M., Borges, F., Garrido, J., 2020.

566   Isothiazolinone biocides: Chemistry, biological, and toxicity profiles. Molecules

567   25.

568 Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D.,

569   2012. Determination of structural carbohydrates and lignin in Biomass. Lab. Anal.

570   Proced. 17.

571 Tucker, M.P., Nguyen, Q.A., Eddy, F.P., Kadam, K.L., Gedvilas, L.M., Webb, J.D.,

572   2001. Fourier transform infrared quantitative analysis of sugars and lignin in

573   pretreated softwood solid residues, in: Applied Biochemistry and Biotechnology.

574   pp. 51–61.

575   Van Bambeke, F., Mingeot-Leclercq, M.-P., Glupczynski, Y., Tulkens, P.M., 2017.

576   Mechanisms of Action, in: Infectious Diseases. Elsevier, pp. 1162-1180.e1.

577   Van Dyk, J.S., Pletschke, B.I., 2012. A review of lignocellulose bioconversion using

578   enzymatic hydrolysis and synergistic cooperation between enzymes-Factors

579   affecting enzymes, conversion and synergy. Biotechnol. Adv.

580   Wahlström, R.M., Suurnäkki, A., 2015. Enzymatic hydrolysis of lignocellulosic

581   polysaccharides in the presence of ionic liquids. Green Chem. 17, 694–714.

582   Wang, J., Kliks, M.M., Jun, S., Jackson, M., Li, Q.X., 2010. Rapid Analysis of Glucose,

583   Fructose, Sucrose, and Maltose in Honeys from Different Geographic Regions

584   using Fourier Transform Infrared Spectroscopy and Multivariate Analysis. J. Food

585   Sci. 75, C208–C214.

586   Ward, B., 2014. Bacterial Energy Metabolism, in: Molecular Medical Microbiology:

587   Second Edition. Elsevier Ltd, pp. 201–233.

588   Williams, T.M., 2006. The mechanism of action of isothiazolone biocides, in: NACE -

589   International Corrosion Conference Series. NACE International, pp. 060901–

590   0609017.

591

592

593

594
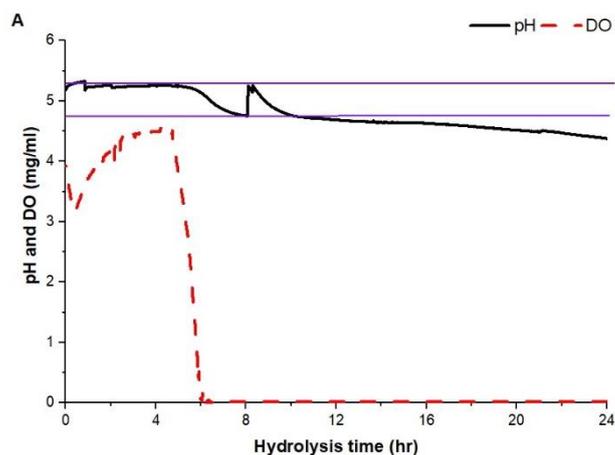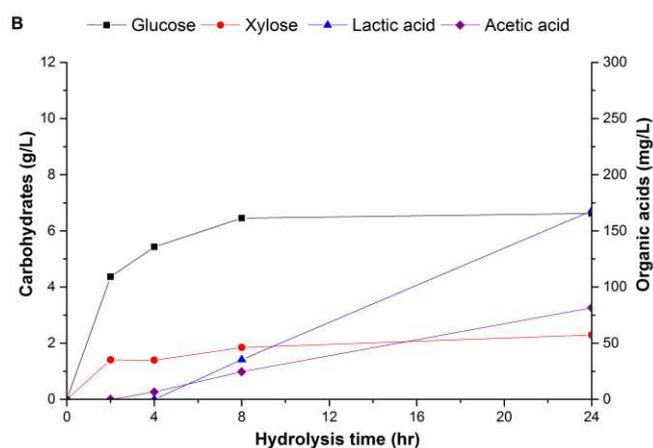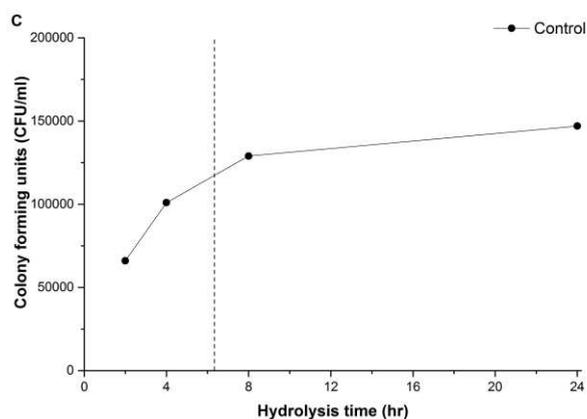
Figure 1. Monitoring of (a) pH and DO, (b) carbohydrates/organic acids, (c) and microbial population density (CFU/ml) over the course of a 24 hour enzymatic hydrolysis of 6% MSW pulp using 2% C-Tec3 (w/w dry substrate) with no antimicrobial supplementation. No pre-sterilization of pulp or equipment was performed. Hydrolysis

615   was carried out at 50 °C, with pH initially set to 5.25, and readjusted at hour 8. Purple

616   lines (Fig. 1a) represent the optimum pH-range (4.75-5.25) for the Cellic® CTec3 enzyme

617   cocktail (Novozymes, 2012)and dashed line (Fig 2c) the timing when DO reached 0 mg/L

618   and anti-microbial was dosed.

619

620

621

622

623

624

625

626

627

628

629

630

631

632

633

634
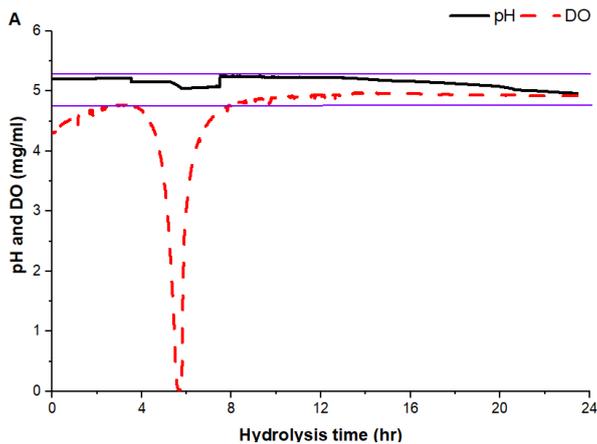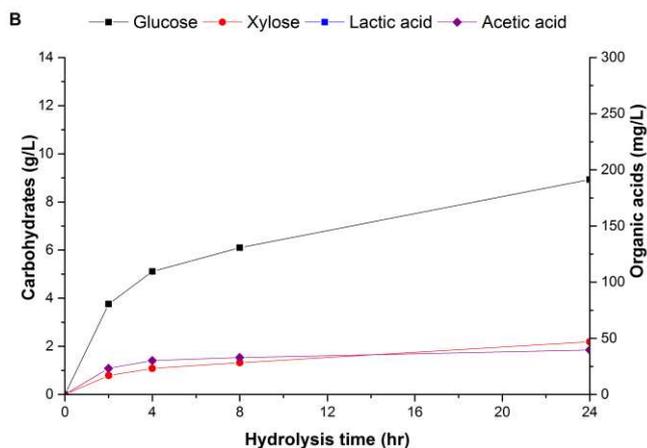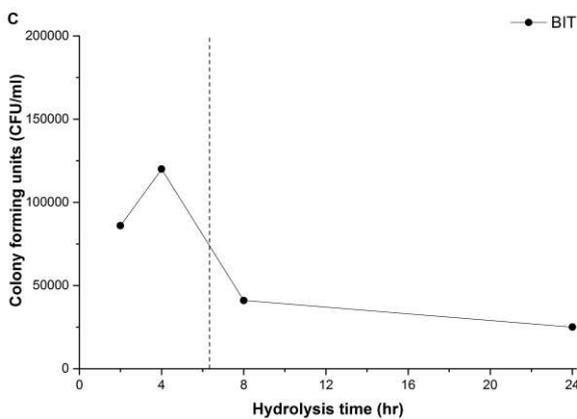
Figure 2. Monitoring of (a) DO and pH, (b) saccharification/fermentation products, and (c) microbial population density (CFU/ml) over the course of a 24 hour enzymatic hydrolysis of 6% MSW pulp using 2% C-Tec3 (w/w dry substrate), where 0.1% BIT (w/w dry substrate) was supplemented into the slurry once DO reached 0 mg/L. No pre-

655 sterilization of pulp or equipment was performed. Hydrolysis was carried out at 50 °C,

656 with pH initially set to 5.25, and readjusted at hour 8. Purple lines (Fig. 1a) represent the

657 optimum pH-range (4.75-5.25) for the Cellic® CTec3 enzyme cocktail (Novozymes,

658 2012) and dashed line (Fig 2c) the timing when DO reached 0 mg/L (anti-microbial

659 dosing).
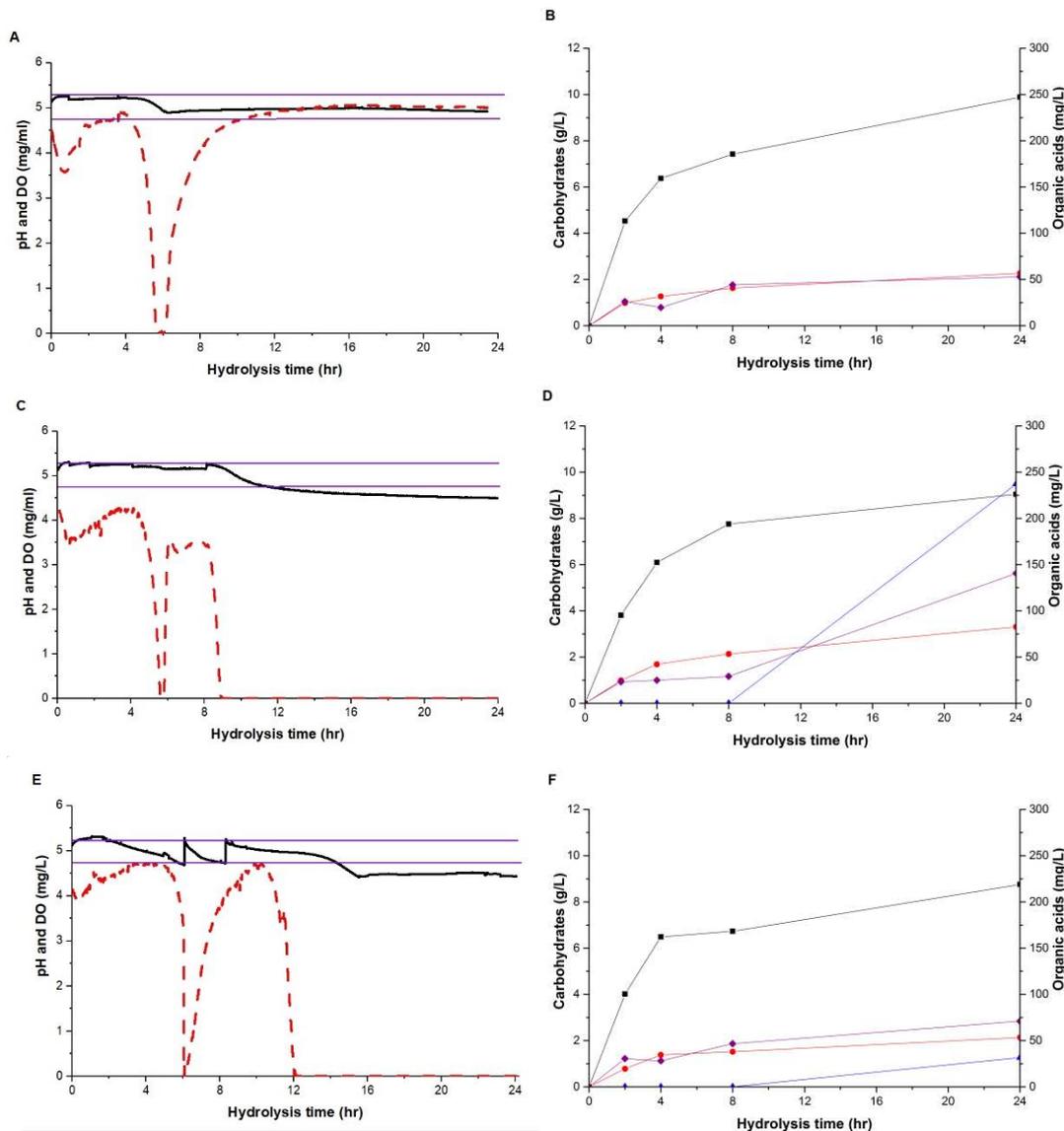
660

661

662

663

664

665

666

667

668

669

670

671

Figure 3. Evaluation of the efficiency of several antimicrobial agents for controlling microbial population during the enzymatic hydrolysis of 6% MSW pulp using 2% C-Tec3 (w/w dry substrate). Graphs on the left pH and DO, graphs on the right carbohydrate and organic acid concentrations. Manual antimicrobial dosing of 0.1% (w/w dry substrate) occurred once DO reached 0 mg/L, with (a,b) sodium azide, (c,d) hydrogen peroxide, and (e,f) tetracycline.   Purple lines (Figs 4a,c,e) represent the optimal pH-range (4.75-5.25) for Cellic® CTec3 enzymes (Novozymes, 2012)
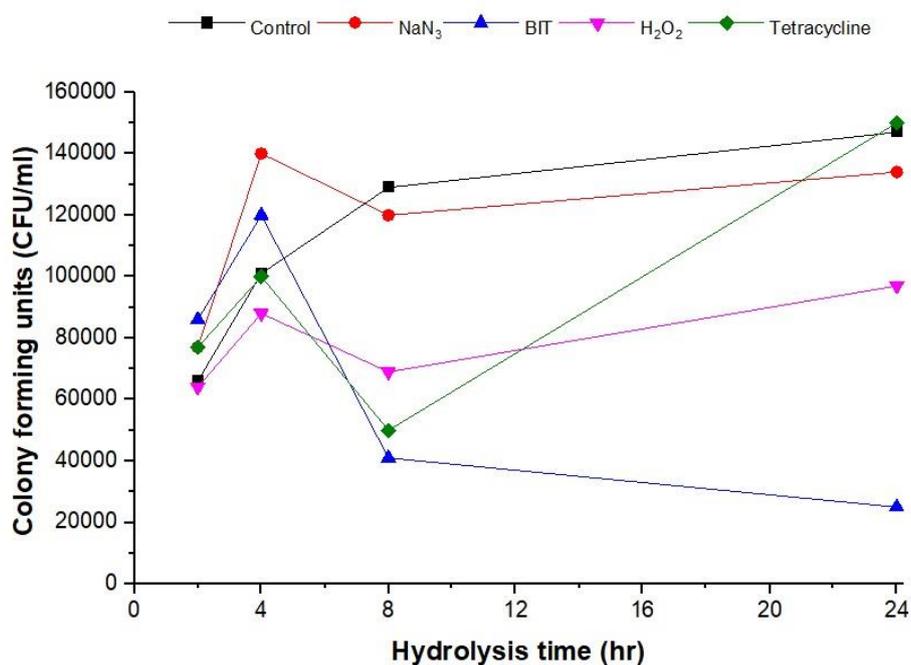
679

680

681

682

683

684

685

686

687 **Figure 4.** Number of colony forming units (CFU) per millilitre of slurry detected at 2, 4,

688 8, and 24 hours from 6% MSW hydrolyses where either 0.1% (w/w dry substrate) BIT,

689 sodium azide, hydrogen peroxide, tetracycline, or no antimicrobial was supplemented

690 into the reaction mixture at approximately hour 6. A serial dilution of slurry at each time

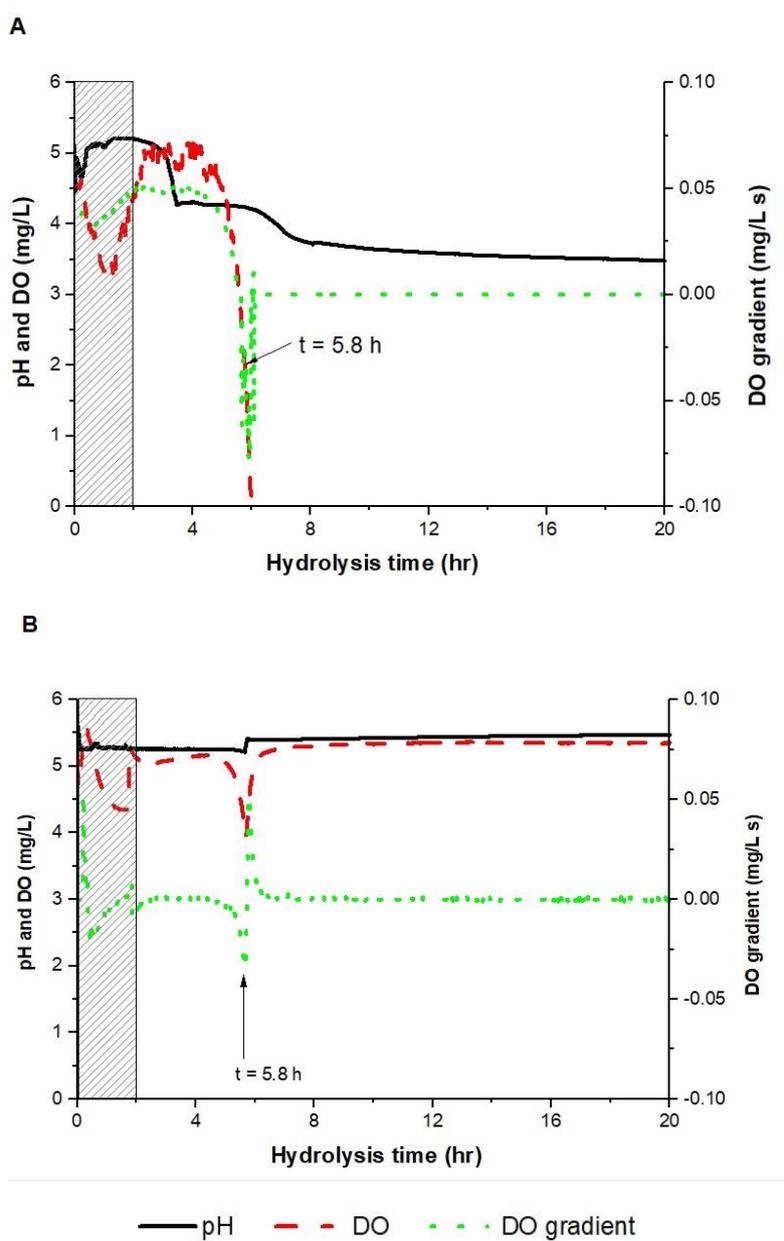691 point was plated onto LB agar and incubated at 50 °C for 16 hours before quantification.

692

**Figure 5**. Process monitoring of pH and DO using automated antimicrobial dosing: (a) control, (b) 0.01% (w/w of dry substrate) NaN_3. Reactions were carried out at 8% TS and 2% E:S. The 30 s readings are plotted as trend-lines, hatched-boxes represent the "viscosity break" and arrows the timing when the DO gradient was -0.028 mg L^{-1} s^{-1}. Final glucose concentrations (t = 20h) were 10.8, 17.2 for the control and NaN_3. Experiments were carried out in duplicate with standard deviations
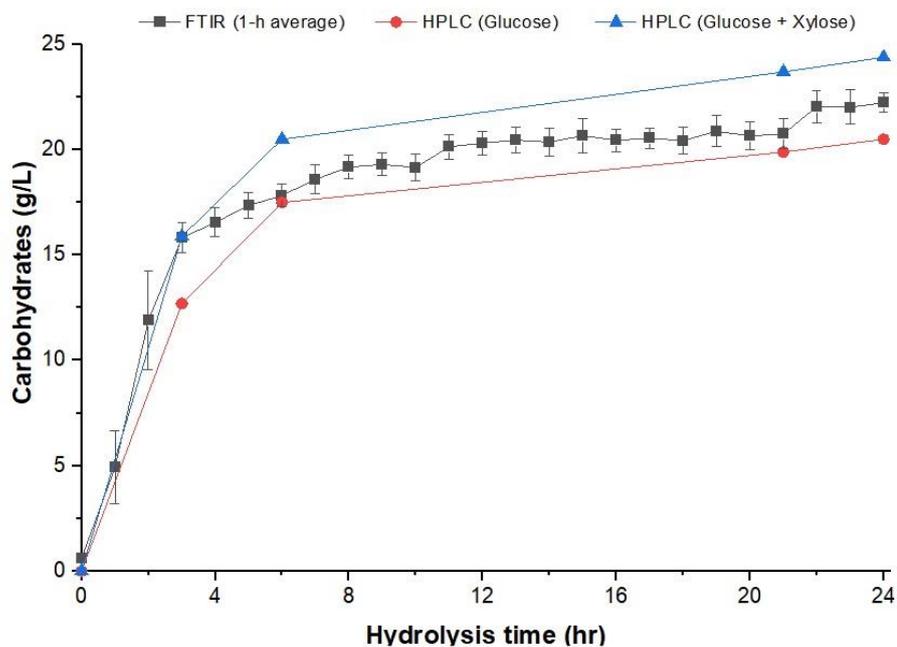
Figure 6. Comparison of continuous FTIR measurement (1-hr averages) and intermittent off-line HPLC measurements (glucose and glucose plus xylose) for the quantification of monomeric sugars during the enzymatic hydrolysis of 7% MSW pulp with 5% C-Tec3 and no antimicrobial supplementation. FTIR values represent the average $\pm$ standard deviation of each measurement taken over the course of an hour.