

1 Enhanced bioproduction of poly-3-hydroxybutyrate from
2 wheat straw lignocellulosic hydrolysates

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13 **Abstract**

14 Polyhydroxyalkanoates (PHAs) are bioplastics that can replace conventional
15 petroleum derived products in various applications. One of the major barriers for
16 their widespread introduction in the market is the higher production costs when
17 compared with their petrochemical counterparts. In this work, a process was
18 successfully implemented with high productivity based on wheat straw, a cheap
19 and readily available agricultural residue, as raw material. The strain
20 *Burkholderia sacchari* DSM 17165 which is able to metabolize glucose, xylose
21 and arabinose, the main sugars present in wheat straw hydrolysates (WSH),
22 was used. Results in shake flask showed that *B. sacchari* cells accumulated ca

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23 70 % g P(3HB)/g cell-dry-weight with a yield of polymer on sugars ($Y_{P/S}$) of 0.18
24 g/g when grown on a mixture of commercial C6 and C5 sugars (control), while
25 these values reached ca 60 % g P(3HB)/g cell-dry-weight and 0.19 g/g,
26 respectively, when WSHs were used as carbon source. In fed-batch cultures
27 carried out in 2L stirred tank reactors on WSH, a maximum polymer
28 concentration of 105 g/L was reached after 61 h of cultivation corresponding to
29 an accumulation of 72% of CDW. Polymer yield and productivity were 0.22 g
30 P(3HB)/g total sugar consumed and 1.6 g/L·h, respectively. The selected
31 feeding strategy successfully overcame the carbon catabolite repression
32 phenomenon observed in sugar mixtures containing hexoses and pentoses.
33 This is the first work describing fed-batch cultivations aiming at PHA production
34 using real lignocellulosic hydrolysates. Additionally, the P(3HB) volumetric
35 productivities attained are, by far, the highest achieved ever on agricultural
36 wastes hydrolysates.

37 **Keywords**

38 Wheat straw hydrolysates ; poly 3-hydroxybutyrate; *Burkholderia sacchari*; carbon
39 catabolite repression; agricultural lignocellulosic residues; high cell density cultures

41 **Introduction**

42 The widespread use of synthetic, petroleum-derived plastics has
43 generated an environmental problem because these materials resist
44 degradation and accumulate in the environment.

45 PHAs are biologically produced macromolecules (polyesters with
46 molecular weights from 5×10^4 to 2×10^6 Da [1]) with a wide range of
47 properties that find applications as biodegradable and biocompatible
48 thermoplastics. They are synthesized by many microbial strains under
49 unbalanced growth conditions such as the presence of excess carbon source
50 and limitation of at least one essential nutrient e.g. phosphorous, nitrogen,

51 sulphur, magnesium or oxygen [2-3]. These polymeric chains are stored in the
52 cytoplasm as granules and function as carbon and energy storage materials.

53 The current high production costs make PHAs more expensive than
54 conventional plastics. In 2011, the prices for PHAs were in the range of 3.7-4.5
55 Euro /kg, while conventional polyolefins such as polyethylene terephthalate and
56 polystyrene were in the range of 1.38-1.63 Euro/kg
57 ([http://www.icis.com/Articles/2011/02/15/9433445/pha-shows-great-promise-in-](http://www.icis.com/Articles/2011/02/15/9433445/pha-shows-great-promise-in-packaging-application.html)
58 [packaging-application.html](http://www.icis.com/Articles/2011/02/15/9433445/pha-shows-great-promise-in-packaging-application.html)). One factor that significantly contributes to the
59 overall PHA production costs is the price of the carbon source [4]. Most of the
60 carbon sources used for PHA production are noble sources such as pure
61 carbohydrates (glucose, sucrose), alkanes and fatty acids. In order to reduce
62 the raw materials costs, inexpensive carbon sources like industrial by-products
63 such as waste glycerol [5], cheese whey [6] and waste plastics [7] or
64 agricultural residues like sugar cane bagasse [8-9], sawdust [10] or forest
65 biomass [11] have been tested as substrates [12]. This approach has the
66 concomitant advantage of converting waste materials into value-added
67 products.

68 Lignocellulosic materials such as agricultural by-products and forestry residues
69 are renewable inexpensive sources of carbohydrates that have no competing
70 food value. These materials consist mainly of cellulose, hemicellulose and
71 lignin. Cellulose and hemicellulose constitute an excellent source of carbon to
72 be used in different biological processes after hydrolysis to monomeric sugars.
73 Cellulose is a highly crystalline linear polymer of β -D-glucopyranose units,
74 joined together in long chains. Hemicellulose is a branched polysaccharide that
75 consists of pentoses, mainly xylose and arabinose, and hexoses such as

76 glucose, galactose and mannose. Cellulose and hemicellulose are embedded in
77 a complex lignin matrix which acts as a binder, conferring to plants structural
78 support, impermeability and resistance against microbial attack and oxidative
79 stress.

80 Agricultural lignocellulosic residues such as wheat or rice straw are abundant
81 feedstocks that have low economic value and are normally used as cattle feed.
82 According to the FAO Cereal Supply and Demand Brief
83 (<http://www.fao.org/worldfoodsituation/wfs-home/csdb/en/>), the world wheat
84 production estimated for the period 2012- 2013 is about 660 million tonnes of
85 which about 15-20 % is straw [14]. Asia and Europe are the primary production
86 regions, with about 43% and 32 %, respectively, while North America is the third
87 largest production region with 15% of global wheat production [15].

88 These agricultural wastes are a potential source of carbohydrates and can thus
89 be upgraded namely in the production of PHAs. In this work, wheat straw
90 hydrolysates (WSH) produced by biorefinery.de GmbH (Teltow, Germany) using
91 the AFEX (Ammonium Fiber Expansion) technology as pre-treatment [16-17],
92 were assayed as carbon source, in the context of the European research
93 project BUGWORKERS (www.bugworkersproject.eu/).AFEX is particularly
94 suited for herbaceous and agricultural residues [18-19], works only moderately
95 well on hardwoods and is not attractive for softwoods [18, 20]. The moderate
96 conditions of the AFEX treatment minimize formation of sugar degradation
97 products [18] such as organic acids (e.g. acetic and formic acid), furaldehydes
98 (e.g. furfural, hydroxymethylfurfural) and aromatic compounds (derived from
99 lignin degradation) which are inhibitory to microbial species.

100 Although the use of lignocellulosic derived carbon sources for the
101 production of biocommodities such as PHAs is an appealing concept, few works
102 are described in literature showing promising results. The economical feasibility
103 of such a system strongly depends on the ability of the strains to consume both
104 C6 and C5 sugars, with high uptake rates, and to accumulate high amounts of
105 PHAs with high yields in a short time. Moreover, in order to achieve high
106 volumetric productivities, a system featuring high-cell-density cultures [2, 5] is
107 essential.

108 In the present work, *Burkholderia sacchari* DSM 17165, a strain able to
109 accumulate PHAs upon consumption of glucose, xylose and arabinose [9, 21],
110 the main sugars present in wheat straw hydrolysates (WSH), was selected. For
111 the first time a fed-batch cultivation process featuring high productivities and
112 conversion yields of P(3HB) based on a lignocellulosic agricultural residue is
113 described.

114 **Materials and Methods**

115 *Microorganisms and media*

116 *Burkholderia sacchari* DSM 17165, a strain able to grow on the main
117 sugars present in the wheat straw hydrolysates and to accumulate PHAs, was
118 used throughout this work.

119 The medium for the seed and flask cultures was (per liter): $(\text{NH}_4)_2\text{SO}_4$, 1.0
120 g; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 4.5 g; KH_2PO_4 , 1.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; yeast extract, 1.0
121 g and a trace elements solution [2], 1.0 mL. The composition of this medium
122 was designed for nitrogen to be the first limiting nutrient. The $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
123 solution was autoclaved separately. The carbon source (noble sugars solution

124 or WSHs) was pasteurized at 70°C for two hours. Sterilization at 121°C was not
125 used to avoid thermal degradation of the sugars. Both the carbon source and
126 the MgSO₄·7H₂O solutions were aseptically added to the medium.

127 The initial medium composition for the fed-batch culture was (per liter):
128 (NH₄)₂SO₄, 4.0 g; KH₂PO₄, 3.0 g; citric acid, 1.7 g; EDTA, 40 mg; trace
129 elements solution [2], 10 mL; MgSO₄·7H₂O, 1.2 g. The pH was adjusted to 6.8
130 with KOH (5N). WSHs or a solution containing a blend of sugars simulating the
131 hydrolysate composition were used as feeding during the fed-batch phase.

132 *Carbon sources*

133 The lignocellulosic hydrolysates were prepared by biorefinery.de GmbH
134 (Teltow, Germany) from grounded wheat straw using the AFEX process as pre-
135 treatment followed by an enzymatic hydrolysis of the cellulose and
136 hemicellulose fractions [22]. The composition of hydrolysate A (Table 1) is
137 representative of the hydrolysates prepared by this methodology. The
138 concentration of inhibitors in the hydrolysates, namely formic and acetic acids
139 and aldehydes such as furfural and hydroxymethyl furfural (HMF) was found to
140 be negligible. Improvement of the WSH composition has been progressively
141 carried out by biorefinery.de GmbH as a direct result of the feedback from the
142 PHA production assays. For instance, the amount of citrate initially used by
143 biorefinery.de GmbH as buffer in the enzymatic step leading to citrate-rich
144 hydrolysates (hydrolysates A and B; Table 1) was substantially reduced (see
145 Results and Discussion) yielding WSH with negligible citrate concentrations
146 (hydrolysates C to H; Table 1). Moreover, the working volume limit of the
147 reactors for PHA production demanded concentrated WSH to be used as

148 bioreactor feed. For this purpose biorefinery.de GmbH has concentrated some
149 of the hydrolysates 10 fold by evaporation (B to H; Table 1).

150 *Strain storage and inoculum preparation*

151 Cultures of *B. sacchari* were stored at -80°C in 2 mL cryovials containing
152 300 µL of glycerol and 1500 µL of a previously grown liquid culture in the late
153 exponential phase prepared with seeding medium [2] and supplemented with 20
154 g/L of xylose. The inocula for the shake flask experiments were prepared by
155 transferring the content of the cryovials to 500 mL shake flasks containing 50
156 mL of seeding medium supplemented with 10 g/L of glucose or xylose, and
157 incubated at 30°C in an orbital incubator (Infors AG, Switzerland) at 170 rpm for
158 12 hours.

159 *Culture conditions*

160 *Shaking flask cultivations*

161 Shaking flask trials were performed in order to determine the growth,
162 consumption and production parameters of *Burkholderia sacchari* DSM 17165
163 using glucose, xylose and arabinose as single sugars or sugar mixtures. The
164 experiments were carried out in 500 mL baffled conical flasks containing 100
165 mL of liquid phase. The inoculum varied in the range 5 to 10 % (v/v) so as to
166 obtain identical initial optical densities (OD_{initial} ca. 0.3). Different concentrations
167 of sugars or blends of sugars were used.

168 Shaking flask experiments were also carried out to compare growth and
169 production on the WSH with those attained on a blend of sugars (control)
170 simulating the sugar composition of the WSH. The trace elements solution, the

171 MgSO₄·7H₂O solution and the WSH or the control were added separately to 500
172 mL flasks to make up a total working volume of 100 mL. The WSHs were
173 neutralized with 5M KOH to a pH of 6.8 prior to use. These assays were
174 performed in duplicate and the average value was considered.

175 *Fed-batch cultivations*

176 Fed-batch cultivations were carried out in 2L stirred-tank reactors (STRs)
177 (New Brunswick Bioflo 115) operated using the BioCommand Batch Control
178 software which enabled control, monitoring and data acquisition. The pH was
179 controlled at 6.8 with 28 % NH₄OH and 2M HCl solutions. The aeration rate
180 used was 3.6 L_{air}/min and the temperature 32 °C. The dissolved oxygen set-
181 point was 20 % saturation and the maximum agitation speed was 1200 rpm.
182 The inoculum (10 % v/v) was prepared using a pair of 500 mL baffled flasks
183 containing 75 mL of seeding medium. Each flask was inoculated with one
184 cryovial, supplemented with 20 g/L of glucose and incubated during 12 h at 30
185 °C and 170 rpm. The initial volume of the fed-batch culture was 1.5 L.

186 Feeding was triggered by the decrease in the stirring speed which resulted
187 from the carbon source exhaustion in the medium. The feeding solution
188 consisted either of a solution of glucose, xylose and arabinose with the same
189 composition of the hydrolysate or the real WSH. To promote polymer
190 accumulation, phosphate limitation was imposed by limiting the initial phosphate
191 concentration in the medium (KH₂PO₄; 3 g/L). Under these cultivation
192 conditions, phosphate became the limiting substrate when the cell concentration
193 reached approximately 35 g/L cell dry weight (CDW). Culture samples were

194 periodically harvested in order to analyze biomass, polymer and sugar
195 concentrations.

196 *Analytical methods*

197 Cellular growth was monitored off-line by measuring the OD of samples at
198 600 nm in a double beam spectrophotometer (Hitachi U-2000). Cell dry weight
199 (CDW) was determined by centrifuging 1.2 mL of culture broth in a Sigma 1-15
200 P microcentrifuge (9168 x g during 4 min) using a previously dried and weighted
201 microtube. The pellet was washed with distilled water and dried at 62 °C in a
202 Memmert oven (Model 400) until constant weight.

203 For P(3HB) determination, 1.2 mL aliquots of culture medium were
204 withdrawn from the broth and centrifuged. The pellet was frozen after being
205 washed with distilled water. This pellet was then subjected to acidic
206 methanolysis [23]. Samples of the organic phase were analyzed in a gas
207 chromatograph (Agilent Technologies 5890 series II) equipped with a FID
208 detector and a 7683B injector. The capillary column was a HP-5 from Agilent
209 J&W Scientific, 30 m in length and 0.32 mm of internal diameter. The oven,
210 injector and detector temperatures were kept constant at 60 °C, 120 °C and 150
211 °C, respectively. Data acquisition and integration were performed by a
212 Shimadzu CBM-102 communication Bus Module and Shimadzu GC Solution
213 software (Version 2.3), respectively. Peak identification was achieved using as
214 standard 3-methyl hydroxybutyrate (Sigma). Calibration curves were obtained
215 using samples of P(3HB) produced previously which were subjected to the
216 same methylation process as the cells.

217 Glucose, xylose and arabinose as well as organic acids, furaldehydes and
218 phosphate concentrations were determined by HPLC (Hitachi LaChrom Elite)
219 equipped with a Rezex ROA-Organic acid H⁺ 8% (300 mm x 7.8 mm) column,
220 an auto sampler (Hitachi LaChrom Elite L-2200), a HPLC pump (Hitachi
221 LaChrom Elite L-2130), a Hitachi L-2490 refraction index detector for sugar and
222 phosphate and a Hitachi L-2420 UV-Vis detector for organic acids and
223 furaldehydes. A column heater for larger columns (Croco-CIL 100-040-220P, 40
224 x 8 x 8 cm, 30°C-99°C) was connected externally to the HPLC system. The
225 injection volume was 20 µL and elution was achieved using a 5 mM solution of
226 H₂SO₄. The column was kept at 65° C and the pump operated at a flow rate of
227 0.5 mL/min.

228 Nitrogen and phosphorous were determined by the methods described in
229 Greenberg, AE. et al, 1992 [24].

230 **Results and Discussion**

231 *Shaking flask cultivations*

232 *Strain selection, growth and P(3HB) production on commercial sugars*

233 Wheat straw contains ca 25 % (w/w) of hemicellulose on a dry weight basis of
234 which ca 80 % are pentoses [25].The economic feasibility of processes using
235 WSHs as carbon sources to produce PHAs strongly depends on the ability of
236 microorganisms to consume both the hexoses and pentoses and to convert
237 these sugars into PHA at high conversion yields ($Y_{P/S}$; g_{polymer}/g_{sugar}) and
238 consumption rates (q_s ; g_{sugar}/g_{cell} ·h). This is crucial both to increase the total
239 carbon up-take by the cells and to avoid pentose accumulation in the broth
240 which may reach inhibitory concentrations.

241 Only few strains have been described in literature as being able to
242 metabolize pentoses and accumulate PHAs. Table 2 gives an overview of the
243 wild and recombinant bacterial strains able to metabolize xylose (the
244 predominant pentose in WSHs) reported so far. Based on these data
245 *Burkholderia sacchari* IPT 101 (*B. sacchari* DSM 17165) a strain able to
246 accumulate high P(3HB) amounts and which shows high yields of polymer on
247 xylose (Lopes et al 2009), was selected to be used throughout this work.

248 Growth and P(3HB) production were followed with *B. sacchari* using
249 different concentrations of glucose (10 and 20 g/L), xylose (10 and 20 g/L) and
250 arabinose (20 g/L) and a mixture of glucose and xylose (10 g/L glucose + 10 g/L
251 xylose). The results for some of these cultivations are shown in Fig. 1. In these
252 assays cell growth occurred until nitrogen in the medium became exhausted
253 and polymer started to accumulate within the cells (at a CDW of approximately
254 3 g/L). For the calculation of the P(3HB) yield on sugars ($Y_{P/S}$; g_{pol}/g_{sugar}),
255 P(3HB) volumetric productivity ($Prod_{vol}$; $g/(L h)$) and P(3HB) cell content ($\%$;
256 g_{pol}/g_{CDW}), the maximum P(3HB) concentration in each assay, its
257 corresponding biomass concentration and total sugar consumption were
258 considered.

259 The results are reported on Table 3. For the single sugar assays using 20
260 g/L glucose, xylose and arabinose, all cultures showed a similar yield of
261 polymer on sugar consumed ($Y_{P/S}$) with a value of ca 0.25 g P(3HB)/g sugar.
262 However, the volumetric productivities are approximately 50 % higher in the
263 case of glucose (0.13 g/(L h)), compared to the productivities on xylose (0.08
264 g/(L h)) and on arabinose (0.09 g/(L h)). Similar results have been reported by
265 Lopes et al, 2009, who claimed that this might be explained by the theoretical

266 ATP/3HB monomer ratio being 3 mol/mol in the case of xylose compared to a
267 value of 7 mol ATP/ mol 3HB in the case of glucose [26]. In sugar mixtures
268 containing 10 g/L glucose and 10 g/L xylose, the strain preferentially consumed
269 glucose and delayed the use of xylose (Fig.1). These results are ascribed to
270 carbon catabolite repression (CCR); i.e., in the presence of sugar mixtures, a
271 preferential consumption of one of the sugars is observed. This is a major
272 problem when dealing with lignocellulosic hydrolysates as fermentation
273 substrates because of incomplete sugar conversion. In some strains CCR is
274 mediated by proteins of the phosphotransferase system (PTS). Lopes *et al.*,
275 2011, studied catabolite repression in PTS mutants of *Burkholderia sacchari*
276 IPT101 in order to improve total carbon up-take in sugar mixtures [27]. The wild
277 strain only started consuming xylose after glucose was completely depleted,
278 while one U.V. mutant was able to consume glucose and xylose simultaneously.
279 At a shaking flask scale, in a medium supplemented with 1 g/L of yeast extract,
280 this mutant showed a specific growth rate, a volumetric productivity and a
281 PHA/carbon yield of 0.43 h^{-1} , $0.12 \text{ g}/(\text{L h})$ and 0.23 g/g , respectively. As
282 compared to its wild strain counterpart, the values were, respectively, 0.41 h^{-1} ,
283 $0.11 \text{ g}/(\text{L h})$ and 0.25 g/g . These authors have studies under way to further
284 overcome catabolite repression in *B. sacchari*.

285 In the present work the efforts have been focused on the development of
286 high cell density fed-batch cultivations of *B. sacchari* DSM 17165 wild strain, at
287 bench-scale, aiming at reaching high volumetric productivities on WSHs. For
288 this purpose, and since the rate of pentose consumption is much affected by the
289 presence of glucose, feeding strategies needed to be sought to enhance the

290 productivity and to avoid the accumulation of xylose and arabinose up to
291 potentially inhibiting concentrations.

292 *Inhibition studies*

293 Inhibition trials were thus carried out to check the inhibitory effect of
294 glucose and xylose concentrations on *B. sacchari* growth and polymer
295 accumulation. Each sugar was tested separately in shaking flasks containing
296 the seeding medium. The maximum specific growth rate (μ_{\max}) was assessed
297 for glucose and xylose in the range of concentrations usually encountered in the
298 bioreactor (10-60 g/L glucose and 10-30 g/L xylose). No growth inhibition was
299 observed up to 60 g/L on glucose (μ_{\max} varied between 0.28 h⁻¹ for 10 g/L
300 glucose and 0.27 h⁻¹ for 60 g/L glucose) and up to 30 g/L on xylose (μ_{\max} varied
301 between 0.21 h⁻¹ for 10 g/L xylose to 0.18 h⁻¹ for 30 g/L xylose).

302 The maximum specific growth rate and P(3HB) production of *B. sacchari*
303 were studied in media containing 10 g/L glucose and increasing concentrations
304 of xylose (0-80 g/L) thus mimicking the accumulation of xylose that could occur
305 in fed-batch cultivations. The assays were followed during 30 h. The maximum
306 specific growth rate was independent of the xylose concentration up to 30 g/L
307 xylose ($\mu \approx 0.30$ h⁻¹) and decreased at higher xylose concentrations. The highest
308 final P(3HB) concentration of 3.8 g/L was obtained at an initial concentration of
309 20-30 g/L xylose. At higher xylose concentrations up to 80 g/L, the final P(3HB)
310 concentration remained constant at approximately 2.0 g/L. After 30 h the
311 glucose present in the medium was completely consumed in all the flasks, while
312 the amount of xylose consumed was constant (about 8.5 g/L) for initial xylose
313 concentrations up to 40 g/L, decreasing for higher xylose concentrations. The

314 highest product yield on C-source was obtained in the absence of xylose (0.24
315 g/g C-source). In the range of 40-80 g/L xylose the yield decreased to a fairly
316 constant value of 0.12 g/g C-source. This observation might be ascribed to a
317 decrease of the pH in the cultivation medium to a value of circa 4.8, which is
318 due to the presence of an unidentified acid (detected by HPLC) accumulating in
319 the medium that apparently inhibits the metabolic activity.

320 This set of experiments indicated that *B. sacchari* DSM 17165 is able to
321 withstand relatively high xylose concentrations without significant loss of
322 activity.

323 *Growth and production on wheat straw hydrolysates*

324 Shaking flask experiments were carried out to study growth and P(3HB)
325 production by *B. sacchari* on WSHs. Hydrolysate A (Table 1) containing 32.4
326 g/L glucose, 12.9 g/L xylose and 4.5 g/L arabinose was tested as C-source and
327 compared to a control (simulated hydrolysate) where the C-source was a
328 mixture of sugars (glucose, xylose and arabinose) with the same concentrations
329 as the hydrolysate. Biomass growth, P(3HB) production and sugar
330 consumptions were followed during the time course of the cultivations. The
331 results are shown in Fig. 2. Production parameters (Table 4) were calculated for
332 each assay based on the maximum polymer concentrations, corresponding
333 biomass concentrations and total sugar consumptions. It is observed that even
334 though similar total biomass concentrations (CDW) were achieved with
335 hydrolysate A and control (CDW = 7.0 g/L and 6.0 g/L, respectively), the total
336 amount of sugars consumed is much lower on hydrolysate A (12.5 g/L) than on
337 the sugar blend (24 g/L). This suggests that on hydrolysate A biomass is

338 preferentially being produced from a carbon source other than sugars. This is
339 reflected in the value of the yield of residual biomass on sugar. Residual
340 biomass (X_{res}) was calculated by the difference of the total biomass dry weight
341 and the concentration of polymer ($X_{res} = CDW - P(3HB)$). Growth on this C-
342 source is however not translated into polymer production, since the amount of
343 P(3HB) produced is proportional to the amount of glucose, xylose and
344 arabinose being consumed and that is reflected on the similar value of yield of
345 polymer on these sugars ($Y_{P(3HB)/sugars} = 0.19 \text{ g/g}$), both for the control and the
346 hydrolysate. This value is similar to the results obtained by Silva et al (2004) for
347 the same strain.

348 These results suggest that there might be other C-sources in the
349 hydrolysate besides sugars which are not used for polymer production. To verify
350 this hypothesis, the hydrolysate was diluted two fold to decrease the
351 concentration of these unknown components. Table 4 shows that upon dilution
352 of the hydrolysate, the total amount of consumed sugars nearly doubled and the
353 same applies to the concentration of P(3HB). These results support the
354 hypothesis of the presence in the WSHs of one or more compounds which are
355 uptaken prior to glucose, xylose and arabinose. One of these compounds is
356 probably citrate which was used as buffer in the enzymatic step of the
357 production of the hydrolysate. Citrate is rapidly consumed since it enters easily
358 the Krebs cycle without the need of being metabolized as the reducing sugars
359 do.

360 *Fed-batch cultivations: preliminary studies*

361 The results and discussion in the previous section confirmed *Burkholderia*
362 *sacchari* DSM 17165 as a good candidate for the bioreactor studies. Stimulation
363 of P(3HB) biosynthesis has been achieved through the limited availability of
364 several nutrients in the medium namely nitrogen, phosphorous, oxygen,
365 magnesium. Previous experiments have been performed to determine which
366 limiting nutrient maximizes polymer accumulation. Higher P(3HB) productivities
367 were obtained when P-limitation was used compared to the N-limitation (data
368 not shown). The combined effect of a larger productivity and the need of less
369 phosphate (lower raw-material costs) was the key for choosing P-limitation to
370 trigger polymer accumulation.

371 *Dynamics of sugar consumption in the STR*

372 In order to better understand the dynamics of sugar consumption in the
373 STR, *B. sacchari* was cultivated fed-batchwise in a synthetic medium containing
374 a mixture of glucose (9 g/L), xylose (8 g/L) and arabinose (2 g/L) as C-sources.
375 The cultures were inoculated with a 24 h grown shake flask culture. Glucose
376 and citrate were consumed preferentially, while xylose and arabinose were only
377 consumed when the glucose concentration was low (Fig. 3). The maximum
378 specific growth rate during the exponential growth phase (before polymer
379 accumulation began).was 0.21 h⁻¹. Approximately twenty hours after
380 inoculation, a 9 mL pulse of a solution containing glucose, xylose and arabinose
381 (250, 200 and 50 g/L, respectively), was manually fed and the sugar
382 consumption was followed. Glucose was consumed rapidly (accompanied by an
383 increased stirring speed, indicating high metabolic activity) while xylose and
384 arabinose consumption proceeded at a lower rate (stirring speed goes to a

385 lower plateau upon glucose exhaustion since less oxygen is needed). Once all
386 sugars have been consumed, the stirring speed drops once again and pH
387 increases rapidly. The sample taken at 21.6 h (the moment at which the
388 agitation decreased to a lower plateau) shows that no glucose was left, while
389 about 10 g/L xylose and 2 g/L arabinose still remained in the medium. Glucose
390 consumption rate was higher than 11 g/(L.h) (all the glucose had been
391 consumed in the 1 h sampling interval), while xylose and arabinose were
392 consumed at a rate of 5.5 g/(L.h) and 2.5 g/(L.h), respectively. At 23 h a
393 second pulse of sugars was added with similar results.

394 To find adequate sugar concentrations to initiate the batch period, two
395 cultivations were started with 30 g/L glucose + 15 g/L xylose and 52 g/L glucose
396 + 26 g/L xylose, respectively. The ratio glucose/xylose chosen was 2 since this
397 is the average ratio present in the WSHs used. The bioreactor was seeded with
398 a 10% (v/v) inoculum grown for 12 h in 20 g/L glucose. A concentrated mixed
399 solution of glucose (440 g/L) and xylose (180 g/L) was manually fed in 50 mL
400 pulses from the moment the initial sugars were depleted until around 12 h of
401 culture. Subsequently automatic feeding of the same solution was switched on
402 (DO-stat). The maximum specific growth rate of the assay that started with less
403 sugar was 0.27 h^{-1} . The assay which was initiated with a higher sugar
404 concentration was probably subjected to substrate inhibition, since a 10 h lag
405 was observed after which the culture also reached a similar maximum specific
406 rate growth rate ($\mu_{\text{max}}=0.28 \text{ h}^{-1}$). To avoid the lag period obtained at higher
407 sugar concentrations, thus resulting in lower productivities, subsequent
408 cultivations were started with lower sugar concentrations (30 g/L glucose, 15
409 g/L xylose and 2.5 g/L arabinose).

410 *Development of feeding strategies*

411 The fed-batch mode of operation was carried out using an automated C-
412 source feeding regime based on the decrease of the stirring speed which
413 happens due to an automatic increase of the dissolved oxygen concentration
414 after C-source exhaustion (DO stat). However, since the rate of glucose
415 consumption is higher than that observed for xylose or arabinose, a drop in
416 stirring speed will immediately occur upon glucose exhaustion, leading to xylose
417 and arabinose accumulation in the cultivation medium. In order to allow for the
418 consumption of the two pentoses, one of two strategies can be adopted, i.e. (i):
419 the stirring speed at which feed is triggered is set at a lower value, allowing for
420 xylose and arabinose consumption to take place, or (ii) the stirring speed trigger
421 is kept high and only glucose is totally consumed, allowing the other two sugars
422 to accumulate before inhibitory concentrations are reached; feeding can then be
423 stopped to allow for complete consumption of xylose and arabinose.

424 Different stirring speed values that trigger automatic feeding were tested
425 and a value of 900 rpm was selected. This value enabled for complete
426 consumption of the glucose in the medium and for partial consumption of xylose
427 and arabinose before another pulse of fresh feed was added. The developed
428 strategy fully succeeded in avoiding the accumulation of xylose and arabinose
429 in the cultivation medium to inhibitory levels.

430 *Cell growth and polymer production in fed-batch cultivations: wheat straw*
431 *hydrolysates versus commercial sugar mixture*

432 To evaluate WSH as a carbon source, *B. sacchari* was thus cultivated in
433 2L controlled stirred-tank reactors (STRs) operated in the fed-batch mode. A

434 control cultivation was carried out using a sugar blend of glucose, xylose and
435 arabinose in which the ratio glucose:xylose:arabinose is the average ratio
436 present in the wheat straw hydrolysates; i.e. 12: 6: 1. The results are shown in
437 Fig 4. The initial sugar composition in the batch phase was 23 g/L glucose, 11.5
438 g/L xylose and 1.9 g/L arabinose. The initial OD of the culture medium was
439 approximately 1.0. Cells grew exponentially until circa 14 h at a μ_{\max} of 0.31 h⁻¹.
440 During the exponential growth phase a 50 mL pulse of feed containing 560 g/L
441 glucose, 280 g/L xylose and 46 g/L arabinose was added. Cells started to
442 accumulate P(3HB) prior to the exhaustion of phosphate in the medium (t=14 h)
443 probably due to oxygen limitation. In fact, in less than 11 h (Fig. 4), the
444 volumetric rate of oxygen consumption was higher than the maximum rate of
445 oxygen transfer to the medium. This can be explained by the high metabolic
446 activity of this strain during glucose uptake and by the maximum allowed stirring
447 speed of 1200 rpm of the bioreactor system. Higher productivities could be
448 obtained if the biomass could grow exponentially before reaching P-limiting
449 values. This would involve strategies such as the use of pure oxygen which are
450 expensive at production scale. After P exhaustion the fermentation proceeded
451 until approximately 40 h when the P(3HB) cell content achieved a constant
452 value of circa 60 % (g P(3HB)/g cell). The yield of polymer on total sugar
453 consumed ($Y_{P/S}$) was 0.17 g/g and the productivity ($Prod_{vol}$) was 1.6 g / (L.h).

454 The first WSH tested in the STR was hydrolysate B (Table 1), an
455 evaporation-concentrated hydrolysate with higher sugar concentrations required
456 for the fed-batch operation. This hydrolysate, containing 468 g/L glucose, 199
457 g/L xylose and 43 g/L arabinose, was used as feed. The same hydrolysate was
458 diluted approximately 25 times to be used as C-source during the batch phase

459 (concentrations of glucose, xylose and arabinose of 18.4, 6.9 and 0.9 g/L,
460 respectively). The cultivation was stopped at 21 hours due to a slowdown of the
461 biomass growth and P(3HB) production (Fig. 5, 1a and 1b). At this point the
462 CDW reached 32.4 g/L and the P(3HB) concentration was 12.2 g/L. The yield of
463 polymer on total sugar consumed ($Y_{P/S}$) was 0.20 g/g, the volumetric
464 productivity ($Prod_{vol}$) was 0.6 g/(L.h) and the polymer accumulated in the cells
465 was 38 % (g P(3HB)/ g CDW). The reason for these deceiving results can be
466 ascribed to the high concentration of citrate present in this WSH (>50 g/L, Table
467 1). Consumption of citrate results in a pH increase of the broth, which in turn
468 interferes with the pH control. During the course of the fermentation, control of
469 the medium pH is achieved by adding ammonia hydroxide (NH_4OH), which is
470 also used to supply nitrogen to the culture. As the fermentation proceeds, the
471 pH increases due to the citrate consumption, resulting in a lack of ammonia, In
472 fact, previous experiments have revealed that when using ammonia limitation in
473 addition to phosphate limitation, both cell growth and polymer production stop
474 (data not shown). During the production of hydrolysates at biorefinery.de,
475 GmbH, citrate was used as buffer for the enzymatic step. Taking into account
476 the above mentioned results, the WSHs producer has subsequently changed
477 the hydrolysis process and was able to supply WSHs containing less than 5 g/L
478 of citrate (hydrolysates C to H on Table 1). Hydrolysate C containing only a
479 citrate concentration of 3.3 g/L was tested next. The initial sugar composition of
480 the medium in the batch phase (23 g/L glucose, 11.7 g/L xylose and 1.9 g/L
481 arabinose) was obtained through dilution of the hydrolysate. Cultivation started
482 with an initial OD of 1.3 (Fig. 5, 2a and 2b). Cells grew exponentially until
483 approximately 15 h with a $\mu_{max}= 0.28 h^{-1}$. After 12 h of cultivation a pulse of feed

484 (hydrolysate C) containing 562.7 g/L glucose, 283.6 g/L xylose and 45.6 g/L
485 arabinose was added, after which the addition of feed proceeded automatically
486 whenever the stirring speed dropped below 900 rpm. At this point, glucose was
487 exhausted, while xylose and arabinose were still present in the cultivation
488 media. Following P- exhaustion, polymer accumulation occurred until the end of
489 the cultivation (approx. 39 h) to a maximum value of 83 g/L, corresponding to an
490 accumulation in the cells of 56 %. The yield of polymer on total sugar consumed
491 ($Y_{P/sugar}$) was 0.20 g/g. The productivity at the end of the cultivation was 1.5
492 g/(L.h). These values are similar to the results obtained with the mixture of
493 commercial sugars used as control. In both cases, after 40 h of cultivation, the
494 consumed xylose is approximately 80 % of the total amount of xylose that was
495 fed whereas this value is circa 95 % for glucose. These figures clearly indicate
496 the ability of the system to promote the consumption of both sugars and
497 circumvent the accumulation of pentoses to inhibitory levels when an
498 appropriate feeding procedure is applied.

499 Very high productivities were attained using these improved hydrolysates
500 compared to the results obtained by other authors. Silva et al. (2004) working in
501 fed-batch conditions with this strain and a blend of glucose and xylose as feed
502 (330 g/L glucose and 360 g/L xylose, mimicking the glucose / xylose ratio
503 present in the bagasse hydrolysates) achieved a biomass concentrations of 60
504 g/L containing 58 % P(3HB) and a maximum P(3HB) productivity of 0.47 g/L.h
505 only. The results presented herein demonstrate (i) the scale-up potential of the
506 developed fed-batch strategy, and (ii) the possibility of achieving high
507 biopolyester productivities based on hydrolysates produced from agricultural
508 residues (WSHs) as carbon source. Moreover, the WSHs produced by

509 biorefinery.de GmbH using the AFEX technology can be directly used as C-
510 source in bacterial cultivations without the requirement of additional processing,
511 namely activated charcoal treatment for the elimination of toxic compounds [9].

512 Further batches of WSH (Table 1, hydrolysates D to H) were finally tested as C-
513 source for growth and P(3HB) production. The results are shown in Fig.6.

514 Maximum cell dry weight and P(3HB) cell content ranged between 100-140 g/L
515 and 45-68%, respectively. The yield of polymer on total sugars consumed and
516 the volumetric productivities varied between 0.16 and 0.22 g/g and between 1.3
517 and 1.5 g/L.h, respectively. The highest productivity (1.6 g/(L.h)) and product
518 yield on sugar (0.22 g/g) were achieved with hydrolysate E. In this cultivation,
519 total xylose and glucose consumptions of 92.0% and 99.5 % respectively, were
520 achieved. The lowest productivities (1.3 g/(L.h)) were obtained with
521 hydrolysates G and H. In these hydrolysates, the ratio of concentrations
522 glucose/xylose varies between 1.2 and 1.4 which is lower compared to the ratio
523 of sugar concentrations found in hydrolysate E (glucose/xylose \approx 2). A lower
524 glucose/xylose ratio influences the frequency of feed additions, i.e. feed
525 triggering becomes less frequent since the overall rate of sugar consumption is
526 lower at higher xylose concentrations. It is however difficult to circumvent
527 process variability when using agriculture-derived C-sources such as WSH,
528 since the composition of these materials depends on a variety of factors,
529 including soil quality, climate and weather conditions, and harvest time.

530 **Conclusions**

531 Fed-batch cultivation strategies of *Burkholderia sacchari* DSM 17165 were
532 developed in order to attain high P(3HB) cell contents and productivities on

533 wheat straw hydrolysates. A polymer cell content of 72 % g/g and a maximum
534 volumetric productivity of 1.6 g/(L.h) were achieved using WSHs rich in glucose,
535 xylose and arabinose as carbon sources in a basal mineral medium. The
536 polymer yield on total sugar consumed ($Y_{P/sugar}$) was 0.22 g/g. At the end of the
537 cultivations, maximum glucose and xylose consumptions were over 99% and 90
538 %, respectively. The proposed feeding procedure was indeed able to overcome
539 the carbon catabolite repression phenomenon associated to the presence of
540 multiple sugars, allowing for an efficient consumption of the pentoses and
541 hexoses present in the hydrolysate.

542 Based on a lignocellulosic agricultural residue, a fed-batch cultivation
543 process for P(3HB) production featuring significantly high productivities and
544 conversion yields is for the first time described.

545

546

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638 **Table 1:** Composition of the different batches of WSH delivered by
639 biorefinery.de GmbH (Germany).






640 **Table 2:** Overview of the strains reported in literature able to metabolize xylose
641 and produce P(3HB).












642 **Table 3:** Cultivation parameters of P(3HB) production by *Burkholderia sacchari*











643 **Table 4:** Growth and P(3HB) production of *B. sacchari* in shake flask cultures
644 on Hydrolysate A, 2 fold diluted Hydrolysate A and simulated Hydrolysate A (all
645 the values refer to the time for maximum polymer concentration).

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







647 **Figure 1:** Growth and P(3HB) production of *B. sacchari* at different sugar
648 concentrations: A: 10 g/L glucose; B: 10 g/L xylose; C: 10 g/L glucose + 10 g/L
649 xylose. CDW (), P(3HB) (), glucose () and xylose ().

650 **Figure 2:** Growth, P(3HB) production and sugar consumption in a) Hydrolysate
651 A, b) mix of commercial sugars and c) 2x diluted Hydrolysate A: CDW (),
652 P3HB (), glucose (), xylose () and arabinose ().

653 **Figure 3:** *B. sacchari* fed-batch cultivation data for growth and production using
654 a blend of glucose (9 g/L), xylose (8 g/L) and arabinose (2 g/L) as C-sources:
655 CDW (), P(3HB) (), glucose (), xylose (), arabinose (),
656 citrate () phosphorous (), nitrogen (), stirring speed, rpm (),
657 % DO (), volume feed, mL ().

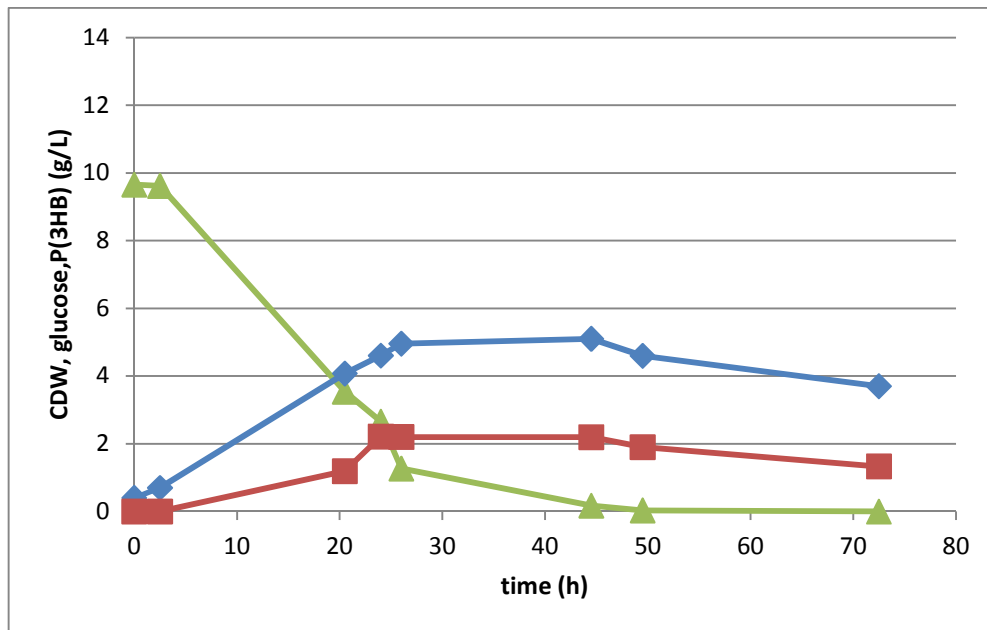
658 **Figure 4:** Cell growth, P(3HB) production, P(3HB) accumulation, consumption
659 of sugars, consumption of phosphate and data acquisition in a 2 L fed-batch
660 cultivation with *B. sacchari* using as feed a blend of sugars. CDW (),
661 P(3HB) (), % P(3HB) (), glucose (), xylose (), arabinose
662 (), phosphate (), % DO (), stirring speed, rpm () and volume
663 feed ().

664

665 **Figure 5:** Cell growth, P(3HB) production, P(3HB) accumulation and sugar,
666 citrate and phosphate consumption by *B. sacchari* in a 2 L fed-batch cultivation
667 using as feed 1a) and 1b): hydrolysate B and 2a) and 2b): hydrolysate C. CDW
668 (), P(3HB) (), % P(3HB) (), glucose (), xylose (),
669 arabinose (), phosphate () and citrate ().

670 **Figure 6:** Cell growth, P(3HB) production and P(3HB) accumulation by *B.*
671 *sacchari* in 2 L fed-batch fermentation using different batches of wheat straw
672 hydrolysate: D (◆), E (■), F (▲), G (✱) and H (●).

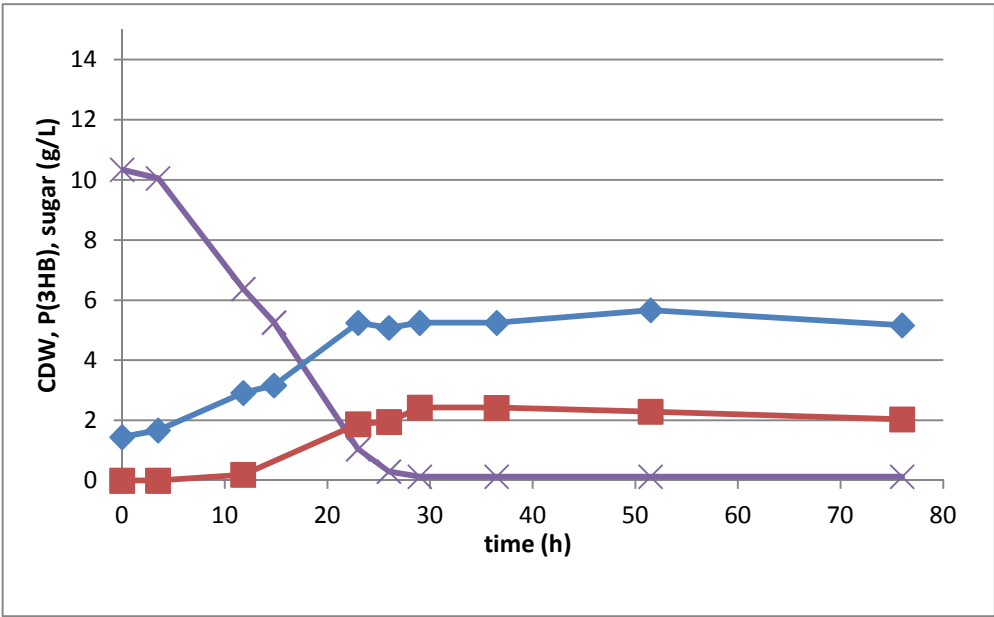
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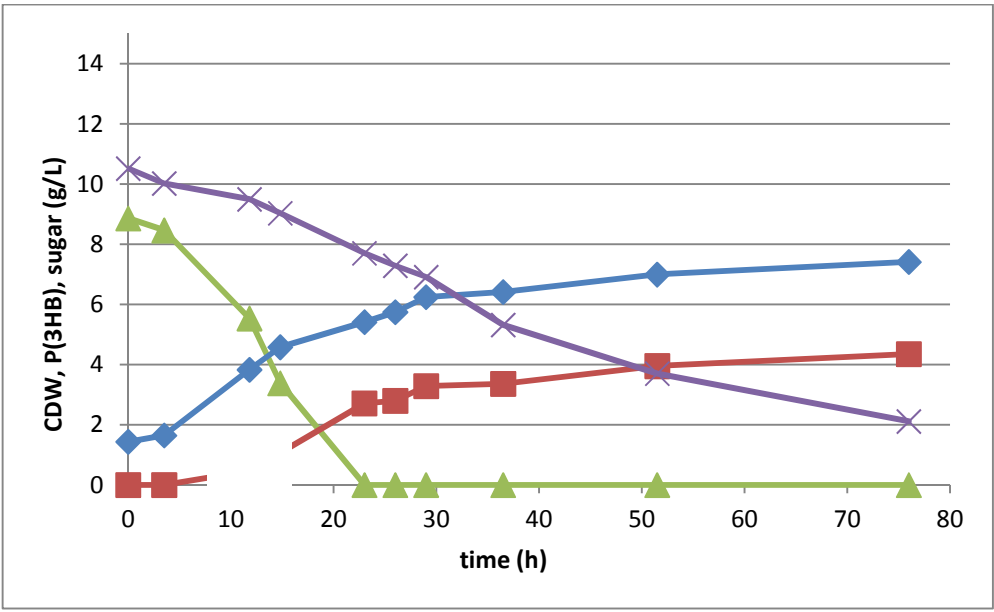
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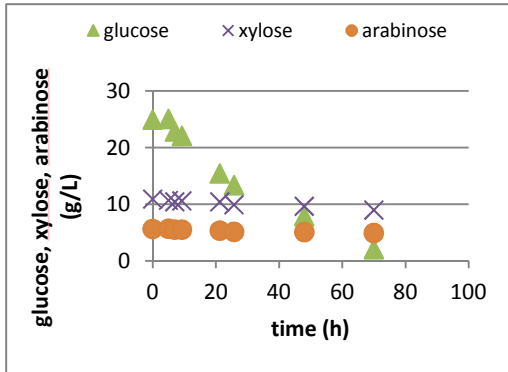
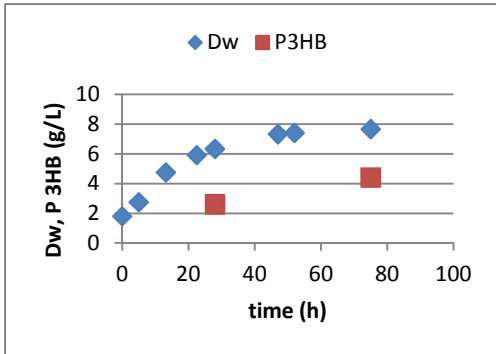


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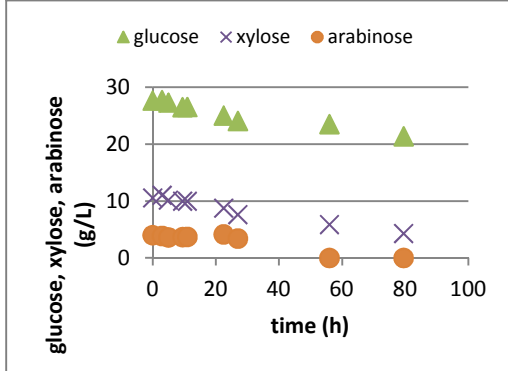
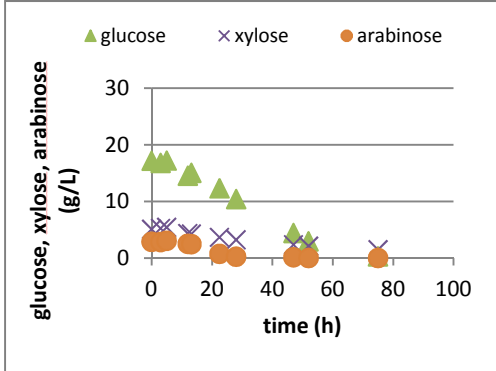
680 Fig 1

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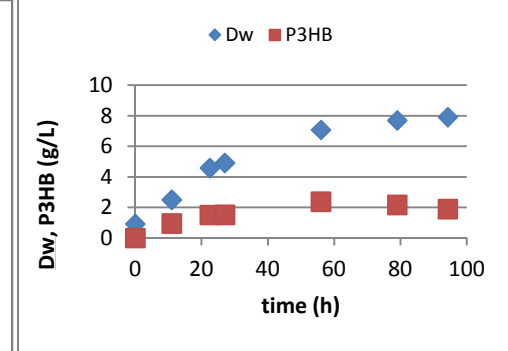
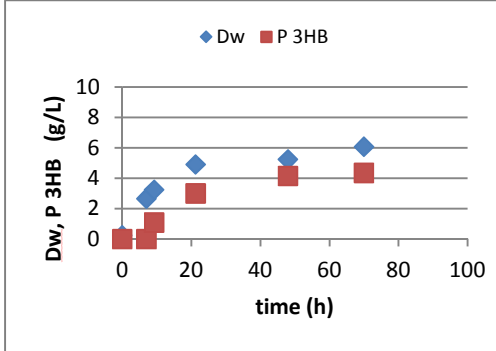
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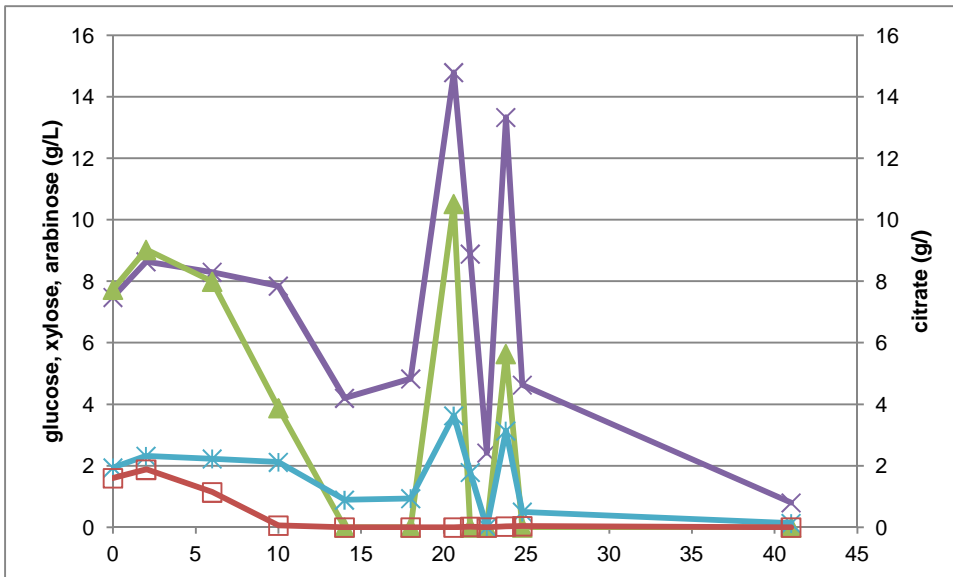
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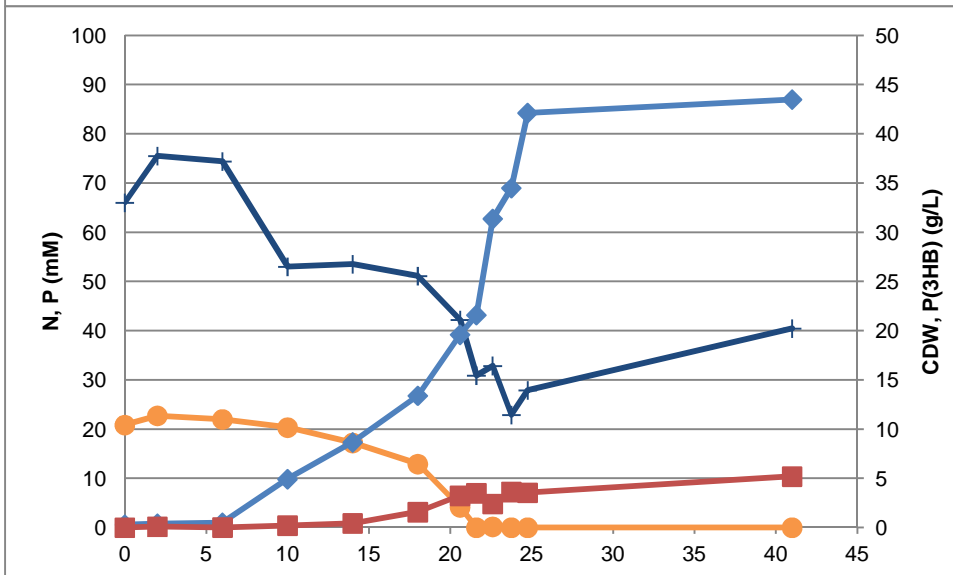
685 Fig 2

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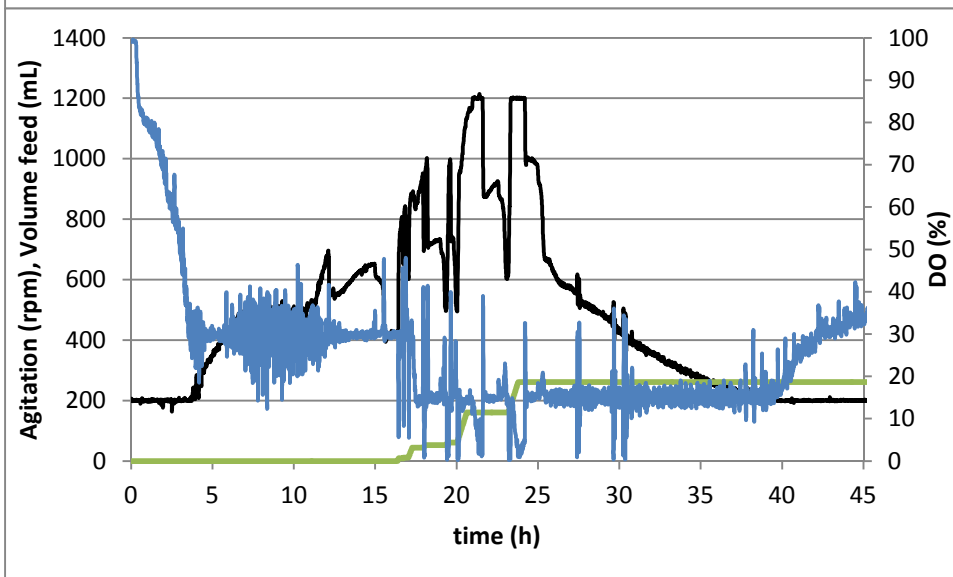
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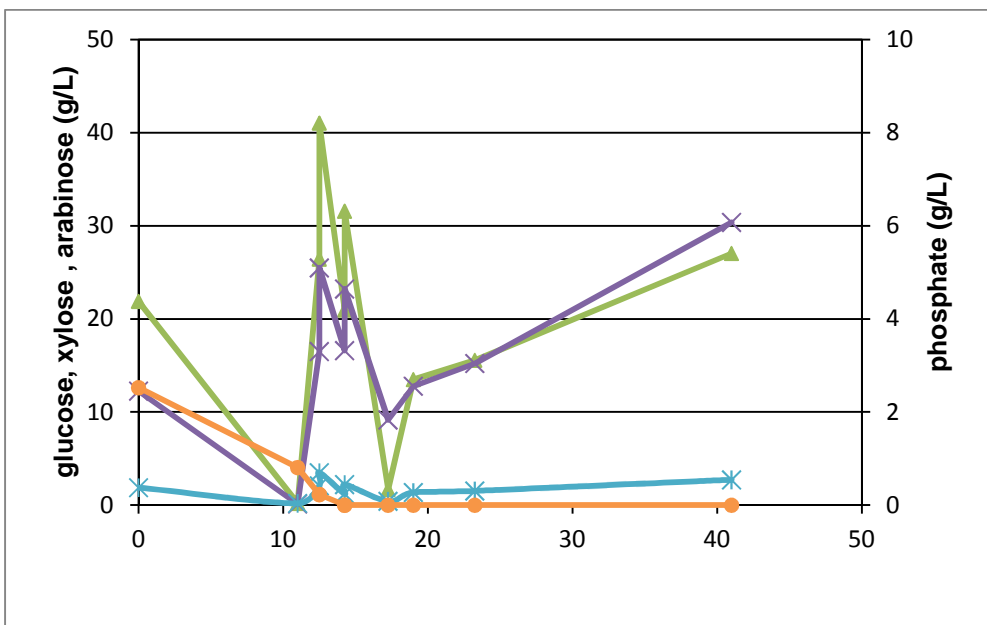
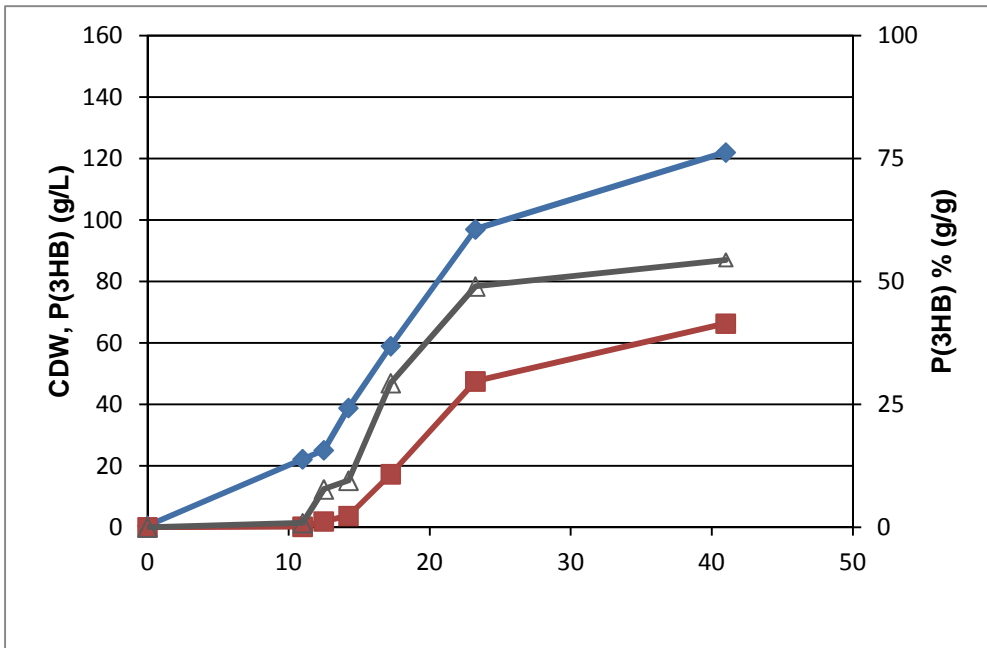
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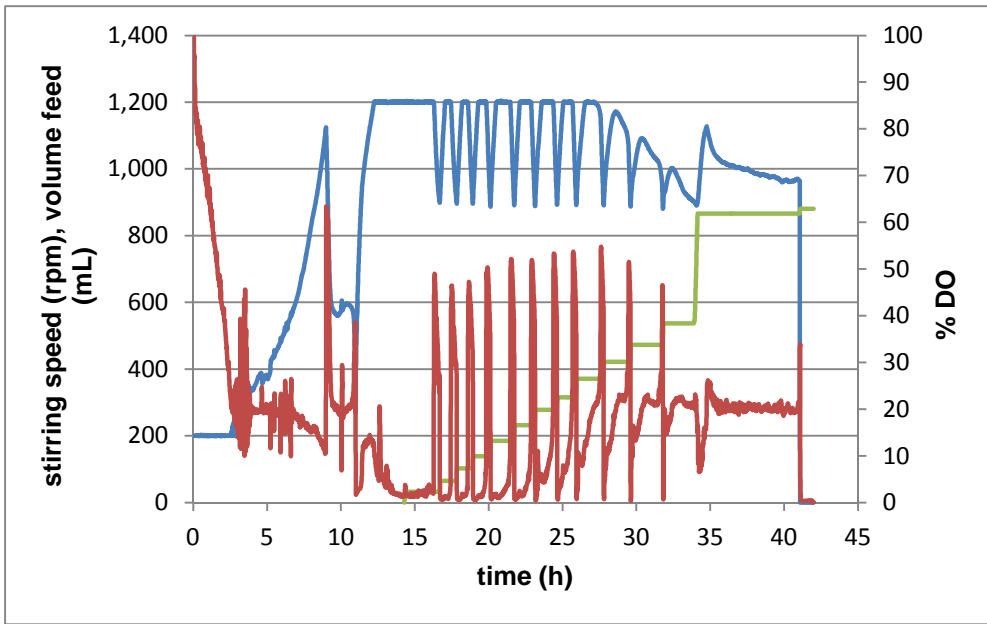


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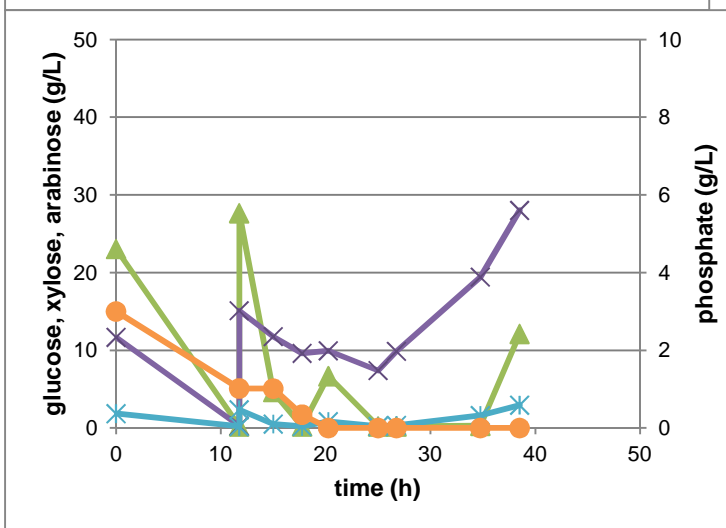
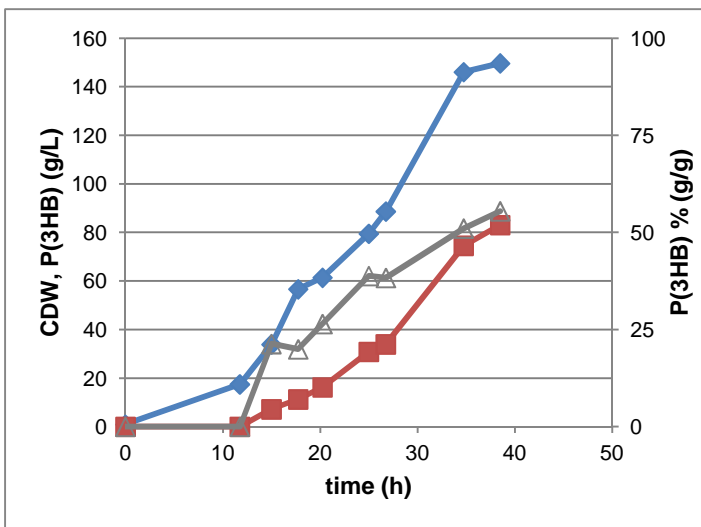


690 Fig 3

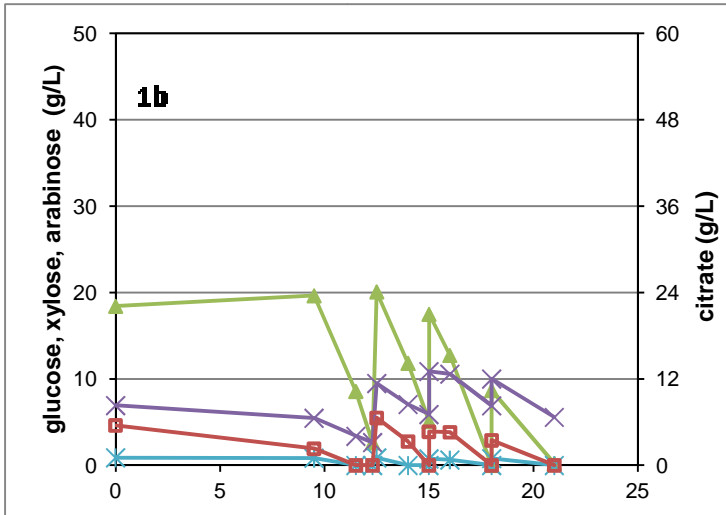




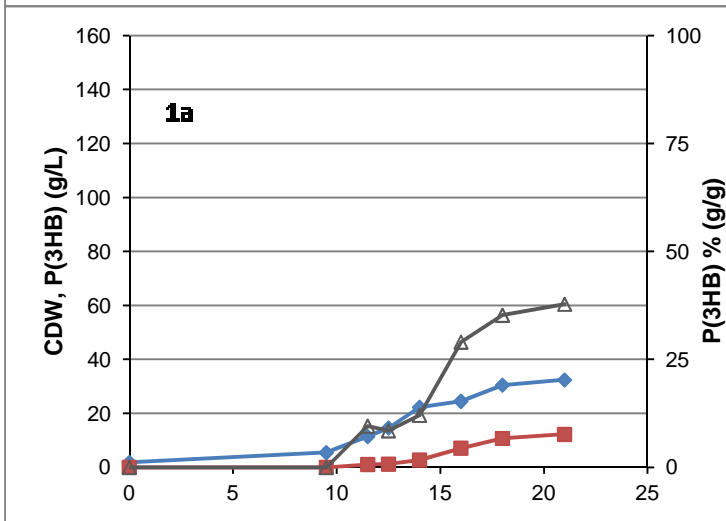
694 Fig 4



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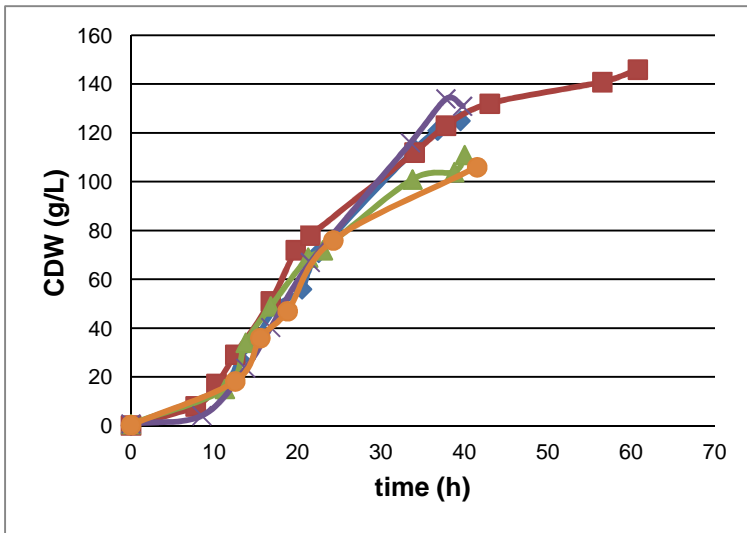


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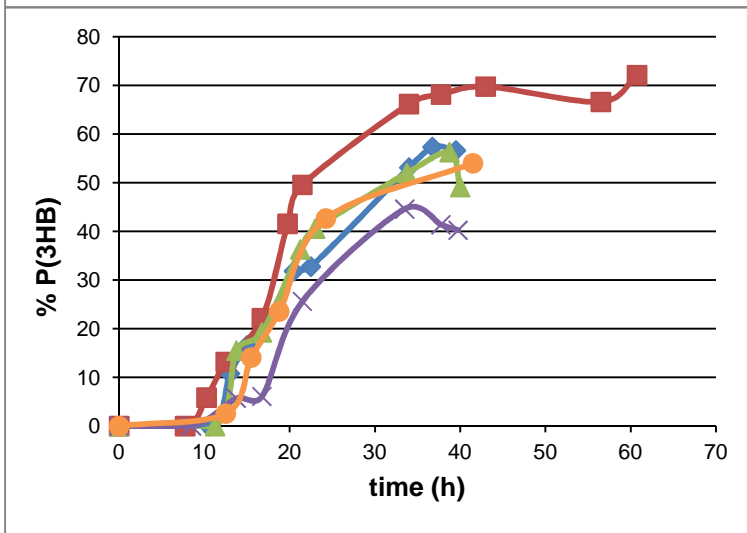


699 Fig 5

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703 Fig 6

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Table 1:

Batches Hydrolysate	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	Furfural (g/L)	Citric acid (g/L)
A	32.4	12.9	4.5	0.10	3.10
B	468.0	199.2	23.2	0.27	57.80
C	562.7	283.6	45.6	0.01	3.30
D	465.3	146.3	41.5	0.03	0.15
E	628.3	314.8	47.6	0.01	0.90
F	445.1	206.1	35.4	ND	0.02
G	485.1	347.0	44.6	0.02	0.25
H	585.0	488.0	42.0	0.02	0.57

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706**Table 2**

Strain	μ_{max} (h ⁻¹)	CDW (g/L)	P(3HB) (%)	Y _{(P3HB)/xyl} (g/g)	q _{P(3HB) max} (g/g·h)	Prod _{vol} (g/L h)	Reference
<i>Burkholderia cepacia</i> ATCC 17759	0.34	7.5	49	0.11	0.02	–	[1]
<i>Burkholderia cepacia</i>	-	-	45	0.11	0.072	-	[2]
<i>Burkholderia sacchari</i> IPT 101	–	5.5	58	0.26	–	0.07	[3]
<i>Burkholderia sacchari</i> LMF828 (mutant PTS ⁻ glu ⁺)	0.35	5.3	50	0.17	–	0.07	[4]
<i>E. coli</i> TG1(pSYL107)	–	4.8	36	–	–	0.028	[5]
<i>Pseudomonas pseudoflava</i> ATCC 33668	0.13	4.0	22	0.04	0.03	–	[6]
Isolated bacterium strain QN271	-	4.3	29	-	-	0.04	[7]

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710 Table 3

Carbon source	conc. (g/L)	time (h)	CDW (g/L)	P(3HB) _{max} (g/L)	Y _{P/S} (g _{P(3HB)} /g _{sugar})	% P3HB (g _{P(3HB)} /g _{CDW})	Prod _{vol} (g _{P(3HB)} /L h)
Glucose	20	29	6.3	3.8	0.25	60.3	0.13
Xylose	20	36.5	6.3	2.8	0.24	44.4	0.08
Arabinose	20	51.5	7.4	4.7	0.24	62.0	0.09
Glucose	10	26	5.0	2.2	0.26	44.0	0.09
Xylose	10	29	5.2	2.4	0.24	46.7	0.08
Gluc + Xyl	10 + 10	76	7.4	4.4	0.25	58.9	0.06

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713 Table 4

Table 4:

Carbon source	ΔS (g/L)	CDW_{max} (g/L)	$P(3HB)_{max}$ (g/L)	X_{res} (g/L)	$Y_{Xres/S}$ (g/g)	$Y_{P/S}$ (g/g)	P(3HB) (%)	time (h)
Hydrolysate A	12.5	7.0	2.4	4.6	0.37	0.19	34	56
Hydrolysate A (2x dil)	22.6	7.7	4.4	3.3	0.15	0.19	57	75
Simulated Hydrolysate A	24	6.0	4.4	1.6	0.07	0.18	72	70

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