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

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Abstract

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

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

Keywords

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

Introduction

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

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

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

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

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

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

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

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

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

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

Materials and Methods

Microorganisms and media

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

The medium for the seed and flask cultures was (per liter): $(NH_4)_2SO_4$, 1.0 g; $Na_2HPO_4.2H_2O$, 4.5 g; KH_2PO_4, 1.5 g; $MgSO_4.7H_2O$, 0.2 g; yeast extract, 1.0 g and a trace elements solution [2], 1.0 mL. The composition of this medium was designed for nitrogen to be the first limiting nutrient. The $MgSO_4.7H_2O$ solution was autoclaved separately. The carbon source (noble sugars solution

124 or WSHs) was pasteurized at 70ºC for two hours. Sterilization at 121ºC was not
125 used to avoid thermal degradation of the sugars. Both the carbon source and
126 the $MgSO_4.7H_2O$ solutions were aseptically added to the medium.

127 The initial medium composition for the fed-batch culture was (per liter):
128 $(NH_4)_2SO_4$, 4.0 g; KH_2PO_4, 3.0 g; citric acid, 1.7 g; EDTA, 40 mg; trace
129 elements solution [2], 10 mL; $MgSO_4.7H_2O$, 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

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

Strain storage and inoculum preparation

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

Culture conditions

Shaking flask cultivations

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

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

$MgSO_4.7H_2O$ solution and the WSH or the control were added separately to 500 mL flasks to make up a total working volume of 100 mL. The WSHs were neutralized with 5M KOH to a pH of 6.8 prior to use. These assays were performed in duplicate and the average value was considered.

Fed-batch cultivations

Fed-batch cultivations were carried out in 2L stirred-tank reactors (STRs) (New Brunswick Bioflo 115) operated using the BioCommand Batch Control software which enabled control, monitoring and data acquisition. The pH was controlled at 6.8 with 28 % NH_4OH and 2M HCl solutions. The aeration rate used was 3.6 L_{air}/min and the temperature 32 ºC. The dissolved oxygen set-point was 20 % saturation and the maximum agitation speed was 1200 rpm. The inoculum (10 % v/v) was prepared using a pair of 500 mL baffled flasks containing 75 mL of seeding medium. Each flask was inoculated with one cryovial, supplemented with 20 g/L of glucose and incubated during 12 h at 30 ºC and 170 rpm. The initial volume of the fed-batch culture was 1.5 L.

Feeding was triggered by the decrease in the stirring speed which resulted from the carbon source exhaustion in the medium. The feeding solution consisted either of a solution of glucose, xylose and arabinose with the same composition of the hydrolysate or the real WSH. To promote polymer accumulation, phosphate limitation was imposed by limiting the initial phosphate concentration in the medium (KH_2PO_4 ; 3 g/L). Under these cultivation conditions, phosphate became the limiting substrate when the cell concentration reached approximately 35 g/L cell dry weight (CDW). Culture samples were

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

Analytical methods

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

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

Glucose, xylose and arabinose as well as organic acids, furaldehydes and phosphate concentrations were determined by HPLC (Hitachi LaChrom Elite) equipped with a Rezex ROA-Organic acid H^+ 8% (300 mm x 7.8 mm) column, an auto sampler (Hitachi LaChrom Elite L-2200), a HPLC pump (Hitachi LaChrom Elite L-2130), a Hitachi L-2490 refraction index detector for sugar and phosphate and a Hitachi L-2420 UV-Vis detector for organic acids and furaldehydes. A column heater for larger columns (Croco-CIL 100-040-220P, 40 x 8 x 8 cm, 30℃-99℃) was connected externally to the HPLC system. The injection volume was 20 µL and elution was achieved using a 5 mM solution of H_2SO_4. The column was kept at 65º C and the pump operated at a flow rate of 0.5 mL/min.

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

Results and Discussion

Shaking flask cultivations

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

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

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

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

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

Inhibition studies

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

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

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

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

Growth and production on wheat straw hydrolysates

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

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

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

Fed-batch cultivations: preliminary studies

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

Dynamics of sugar consumption in the STR

In order to better understand the dynamics of sugar consumption in the STR, *B. sacchari* was cultivated fed-batchwise in a synthetic medium containing a mixture of glucose (9 g/L), xylose (8 g/L) and arabinose (2 g/L) as C-sources. The cultures were inoculated with a 24 h grown shake flask culture. Glucose and citrate were consumed preferentially, while xylose and arabinose were only consumed when the glucose concentration was low (Fig. 3). The maximum specific growth rate during the exponential growth phase (before polymer accumulation began).was 0.21 h^{-1}. Approximately twenty hours after inoculation, a 9 mL pulse of a solution containing glucose, xylose and arabinose (250, 200 and 50 g/L, respectively), was manually fed and the sugar consumption was followed. Glucose was consumed rapidly (accompanied by an increased stirring speed, indicating high metabolic activity) while xylose and 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 (μ_{max}=0.28 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).

Development of feeding strategies

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

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

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

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

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

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

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

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

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

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

Further batches of WSH (Table 1, hydrolysates D to H) were finally tested as C-source for growth and P(3HB) production. The results are shown in Fig.6. Maximum cell dry weight and P(3HB) cell content ranged between 100-140 g/L and 45-68%, respectively. The yield of polymer on total sugars consumed and the volumetric productivities varied between 0.16 and 0.22 g/g and between 1.3 and 1.5 g/L.h, respectively. The highest productivity (1.6 g/(L.h)) and product yield on sugar (0.22 g/g) were achieved with hydrolysate E. In this cultivation, total xylose and glucose consumptions of 92.0% and 99.5 % respectively, were achieved. The lowest productivities (1.3 g/(L.h)) were obtained with hydrolysates G and H. In these hydrolysates, the ratio of concentrations glucose/xylose varies between 1.2 and 1.4 which is lower compared to the ratio of sugar concentrations found in hydrolysate E (glucose/xylose≈ 2). A lower glucose/xylose ratio influences the frequency of feed additions, i.e. feed triggering becomes less frequent since the overall rate of sugar consumption is lower at higher xylose concentrations. It is however difficult to circumvent process variability when using agriculture-derived C-sources such as WSH, since the composition of these materials depends on a variety of factors, including soil quality, climate and weather conditions, and harvest time.

Conclusions

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

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

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

Acknowledgements

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

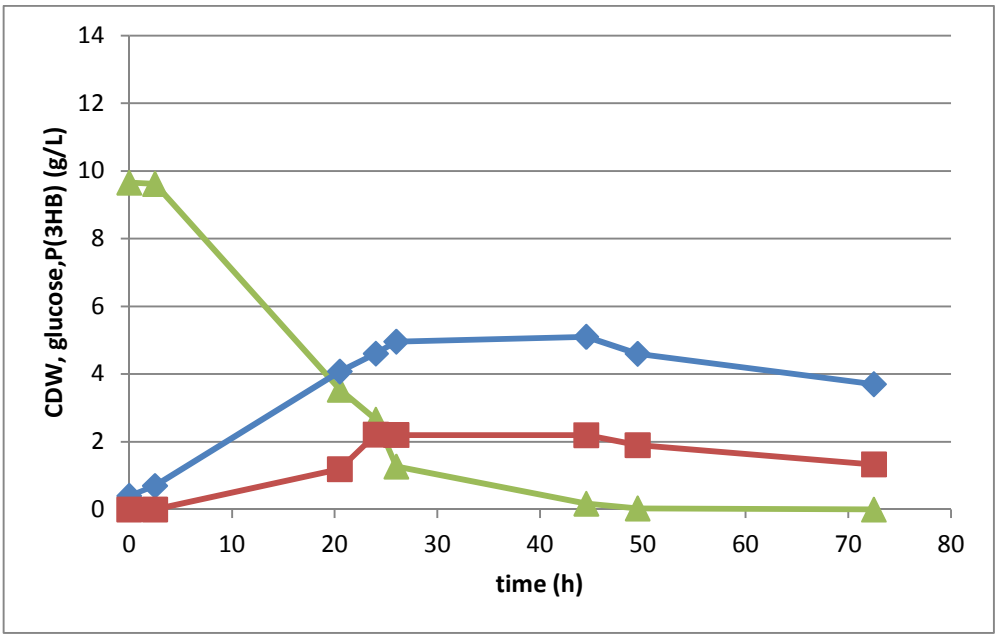
Figure 2: Growth, P(3HB) production and sugar consumption in a) Hydrolysate A , b) mix of commercial sugars and c) 2x diluted Hydrolysate A:CDW ( ), P3HB ( ), glucose ( ), xylose ( ) and arabinose ( ).

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

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

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

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



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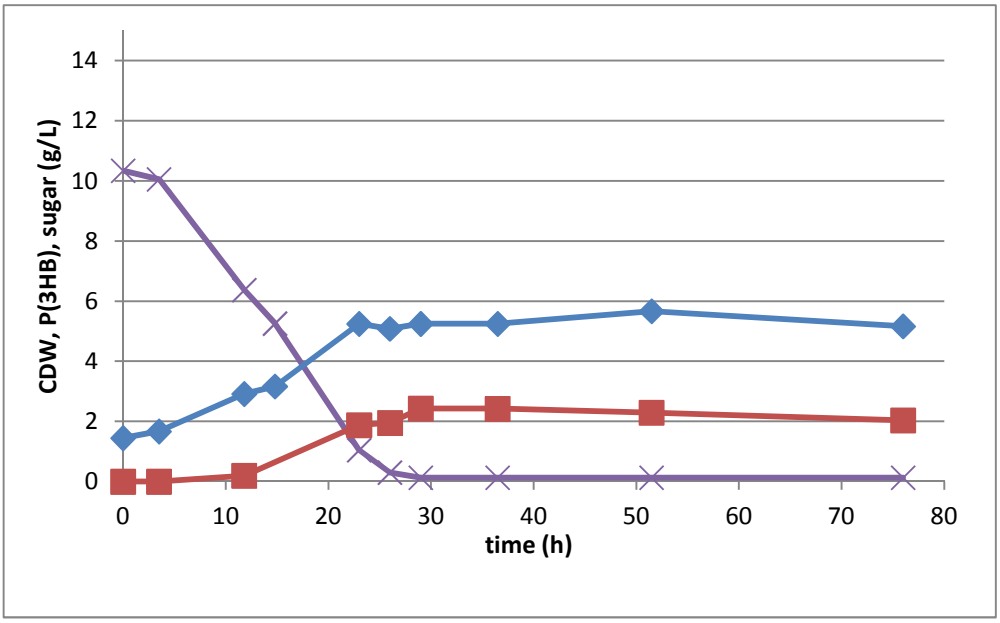

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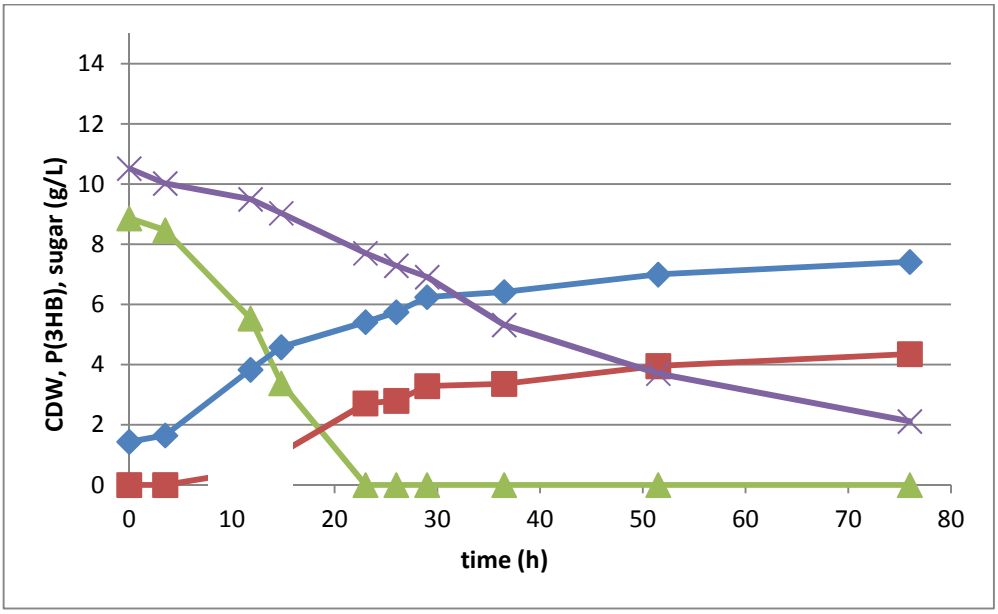

680 Fig 1

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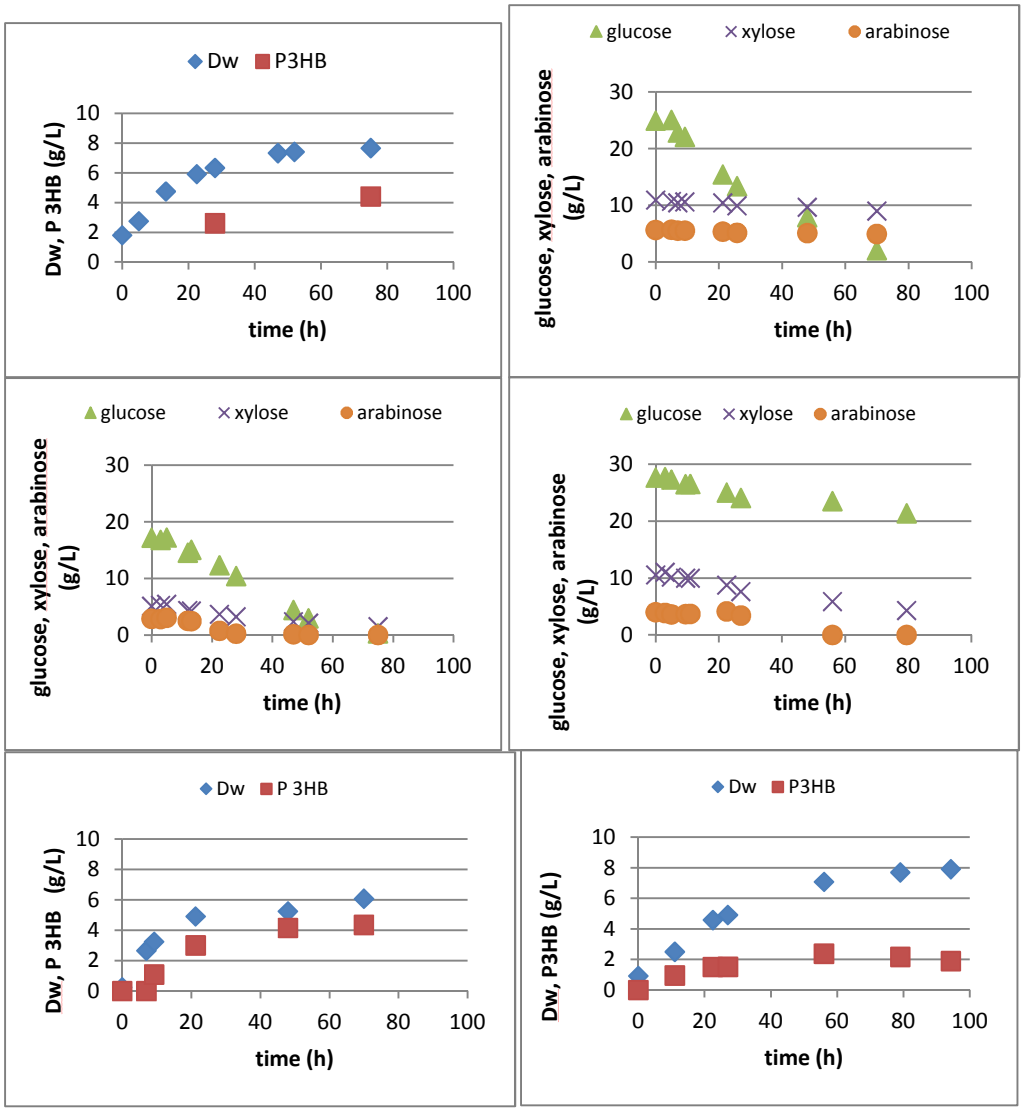

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685 Fig 2

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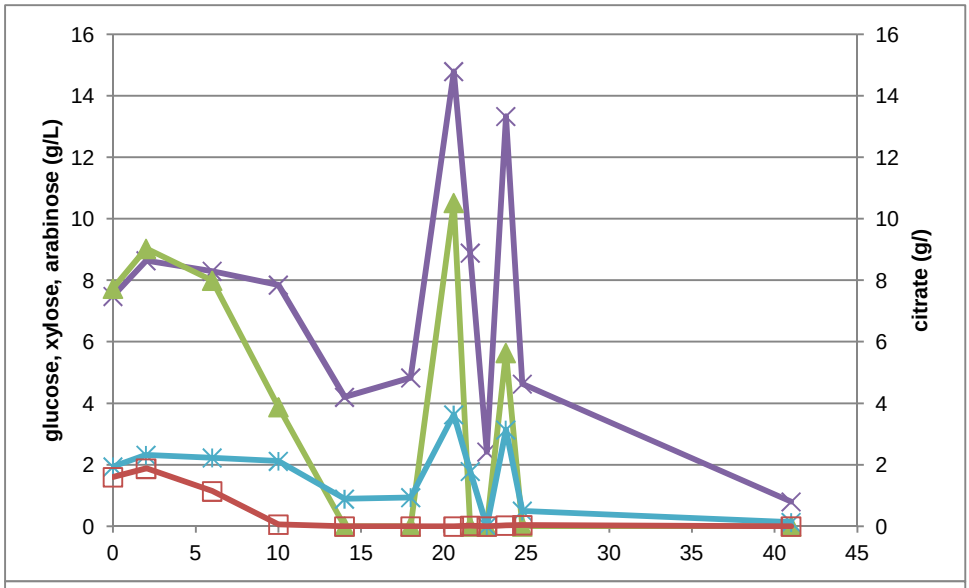

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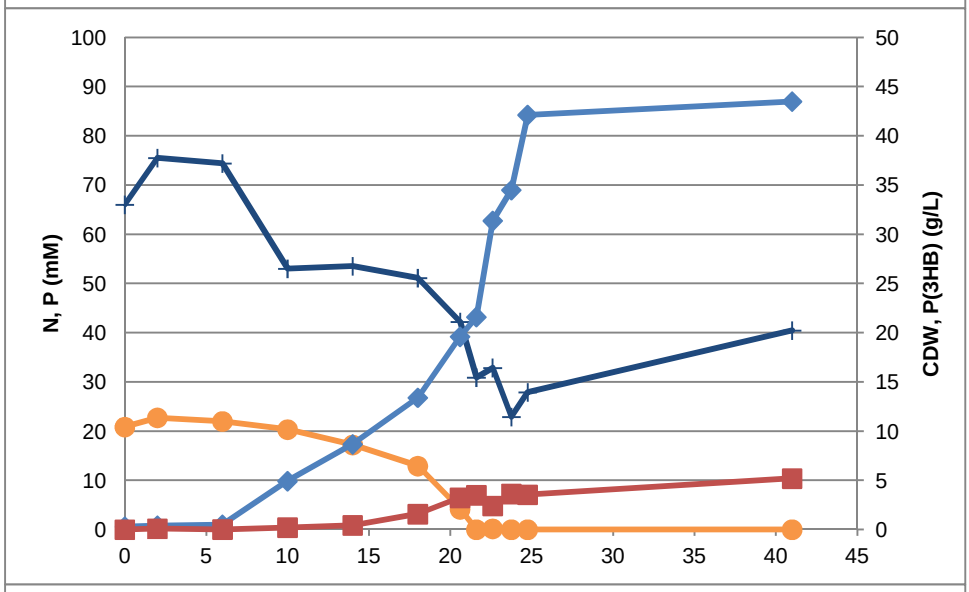

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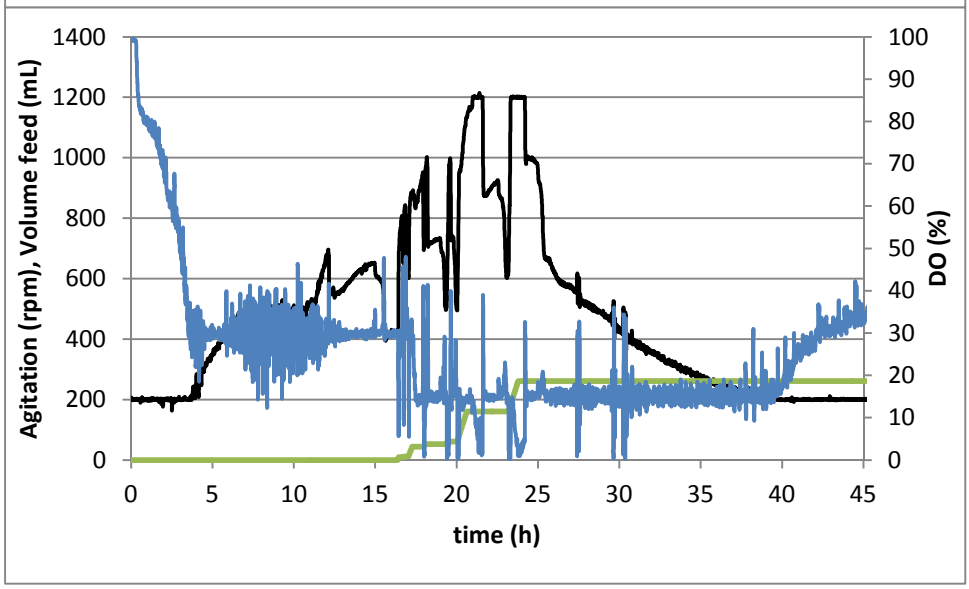
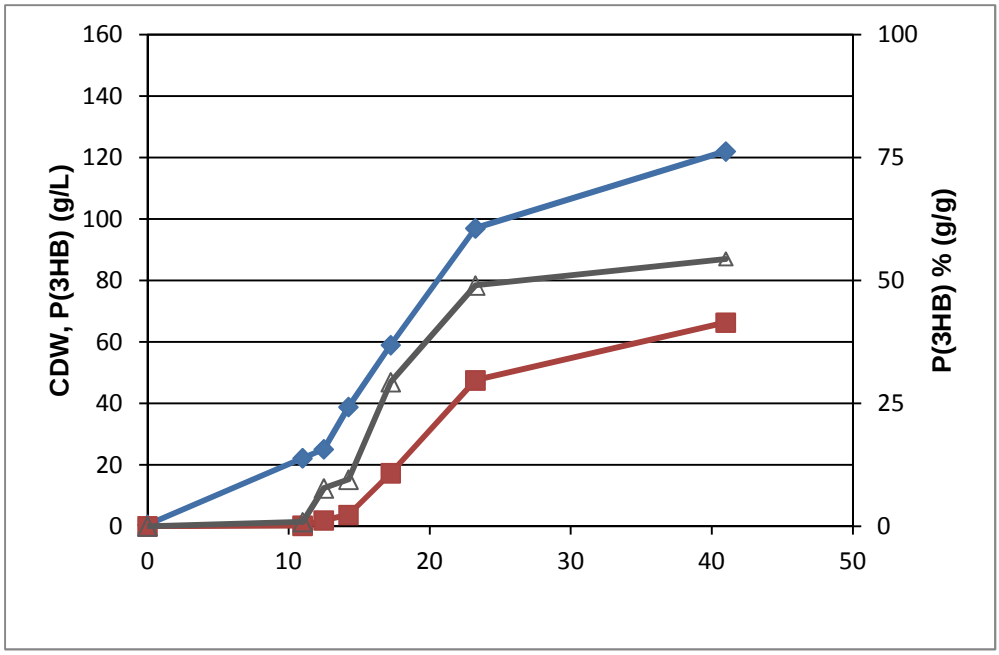


690 Fig 3


160
140
120
100
80
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20
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CDW, P(3HB) (g/L)
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P(3HB) % (g/g)
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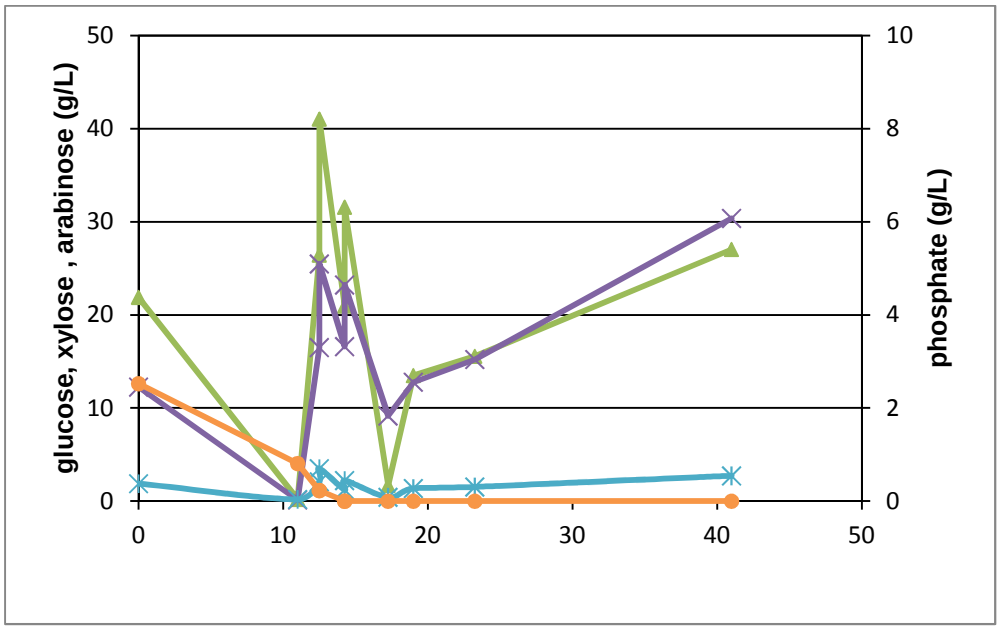
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40
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glucose, xylose , arabinose (g/L)
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phosphate (g/L)
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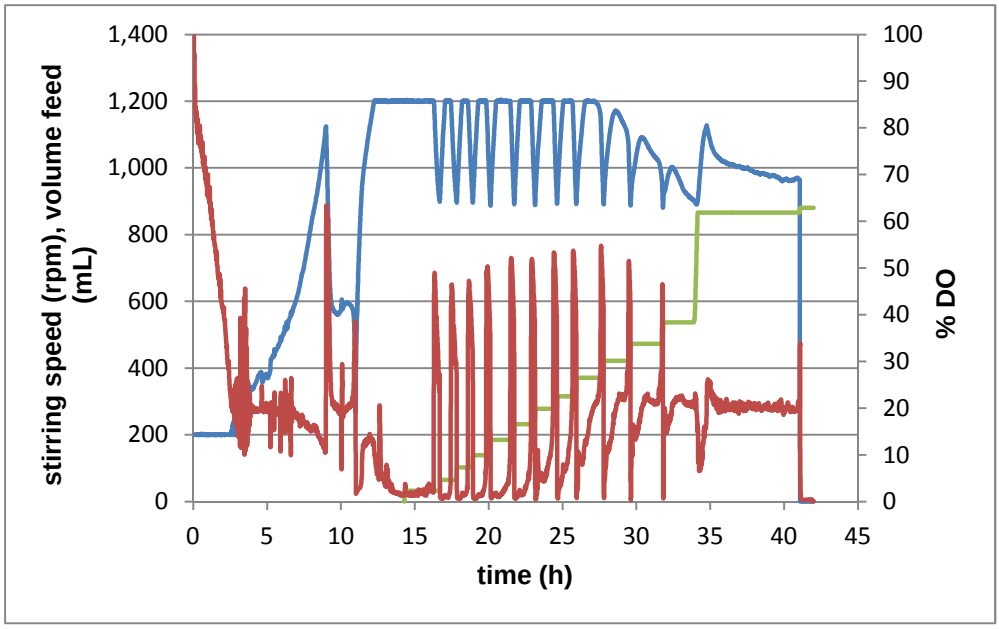

694 Fig 4

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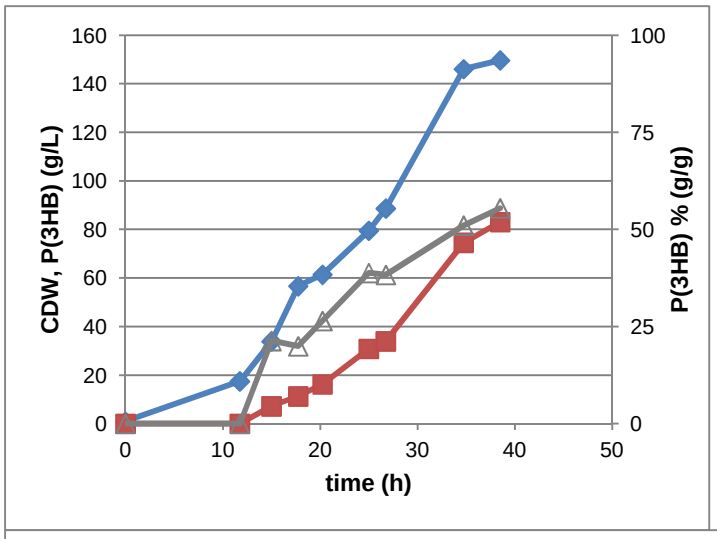

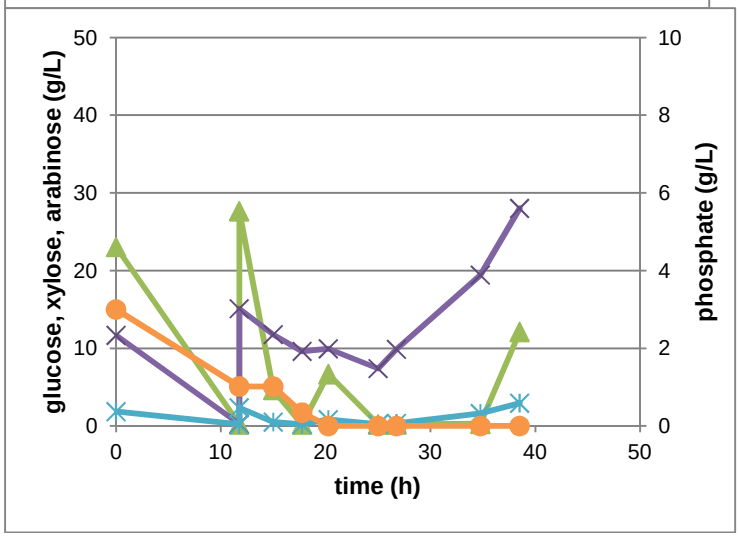

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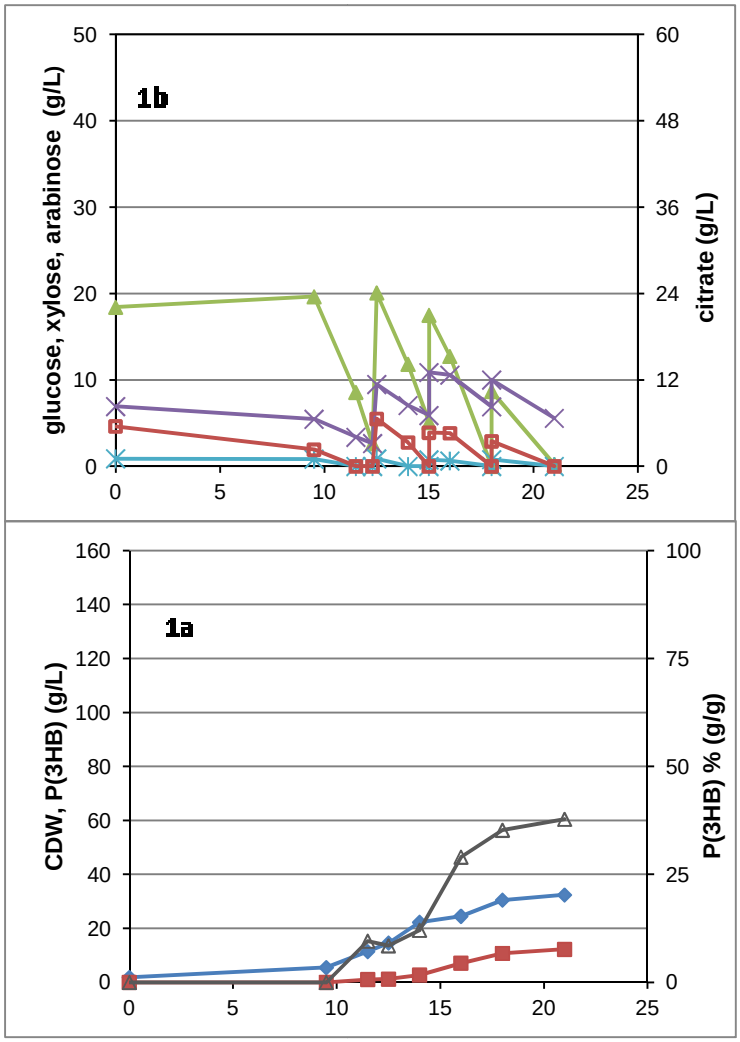

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699 Fig 5

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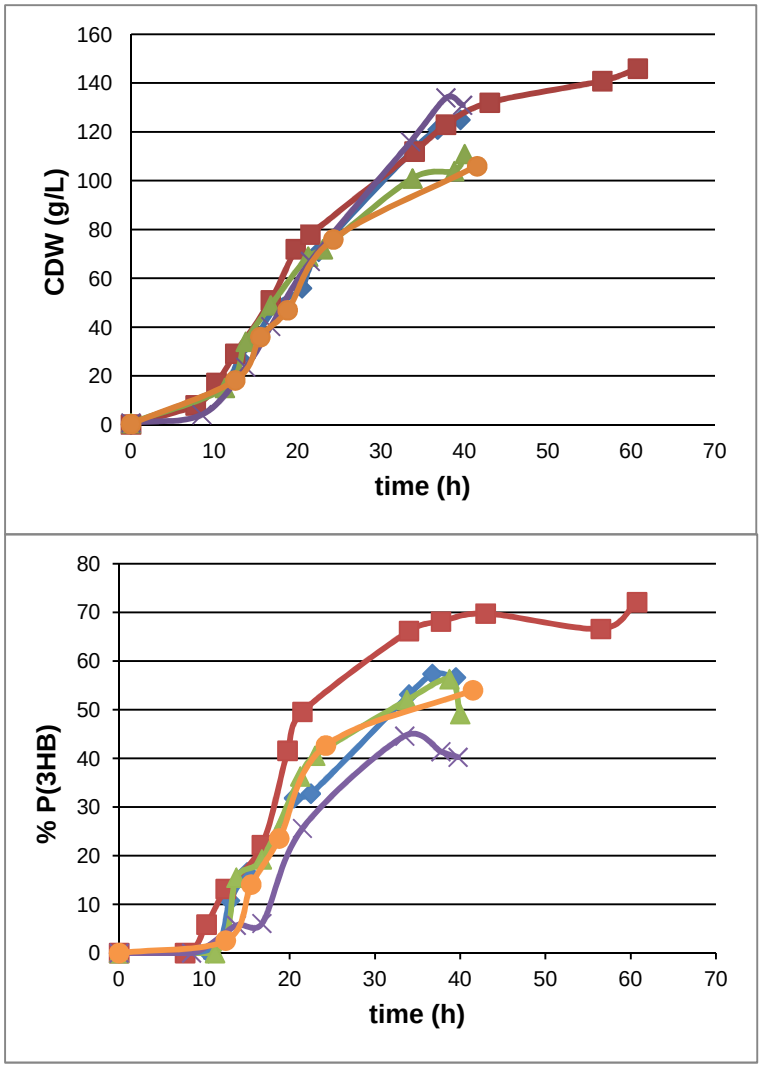

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703 Fig 6

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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

705
706 Table 2

Strain	μmax (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
Burkholderia cepacia ATCC 17759	0.34	7.5	49	0.11	0.02	_	[1]
Burkholderia cepacia	-	-	45	0.11	0.072	-	[2]
Burkholderia sacchari IPT 101	_	5.5	58	0.26	_	0.07	[3]
Burkholderia sacchari LMF828 (mutant PTS^- glu+)	0.35	5.3	50	0.17	_	0.07	[4]
E. coli TG1(pSYL107)	_	4.8	36	_	_	0.028	[5]
Pseudomonas pseudoflava 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

711
712

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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