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Production of butyric acid by *Clostridium tyrobutyricum* (ATCC25755) using sweet sorghum stalks and beet molasses

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ABSTRACT

Enzymatically liquefied sweet sorghum stalks and beet molasses were evaluated for butyrate production using *Clostridium tyrobutyricum* in 1 L scale fed-batch fermentations. The hydrolysates used for the fermentations were prepared separately by liquefying the sorghum stalks at 50 °C, pH 5.0 for 18 h, with 30% (w/v) DM content using the enzyme preparation Cellic® CTec2 at an activity of 26.5 FPU/g DM. To enhance sucrose consumption, the fermentations were supplemented with invertase at an activity equivalent to 8.3 U/g DM. With the hydrolysate as the feedstock, a butyrate concentration of 37.2 ± 0.8 g/L, a productivity of 0.86 ± 0.02 g/L h and a yield of 0.39 ± 0.02 g/g ($p = 0.05$) consumed sugars were obtained. Finally, a butyrate concentration of 58.8 g/L, a productivity of 1.9 g/L h, a butyrate yield of 0.52 g/g consumed sugars and a dry cell mass concentration of 15.7 g/L were obtained with fed-batch cultivation and a constant feed consisting of 64% sorghum hydrolysate juice and 36% molasses. Evidence for inducible saccharolytic activity was also proven, as the cellulase activity in the culture supernatant was found more than double during feed with limiting sugar concentrations. The present study clearly demonstrates that combinations of low cost raw materials can be used for efficient butyrate production, also without cell immobilization.

1. Introduction

Butyric acid has important applications in the solvent, polymer and specialty chemical market and is generally derived from the petrochemical industry by the oxy process (Mascal, 2012). However, environmental concerns have revived the interest for using renewable resources and microbial fermentation technologies for the production of high-added value fuels and chemicals (Koutinas et al., 2014). Several avenues for producing biofuels and solvents from butyrate have been investigated. For example, butanol which is a promising biofuel and an important solvent can be produced by catalytical reduction of biologically derived butyrate with hydrogen (Ju et al., 2010). Ethanol and butanol derived from fermentation technologies can be enzymatically esterified with butyrate to produce ethyl-, and butyl butyrate (Van den Berg et al., 2013). Butyl butyrate has been considered as an alternative aviation fuel (Chuck and Donnelly, 2014).

The highest reported butyrate productivities and product concentrations have been obtained with the anaerobic bacterium *Clostridium tyrobutyricum* (Jiang et al., 2011). Several different cultivation techniques have been investigated for butyrate production including batch, (Fayolle et al., 1990), fed-batch (Mitchell et al., 2009) and continuous systems (Du et al., 2014). Batch fermentations have been reported to give butyrate titers of up to 62.5 g/L, volumetric productivities of 0.99 g/L h and butyrate yields of 0.466 g/g with glucose as carbon source (Song et al., 2010). However, these fermentation results were obtained with glucose concentrations of up to 150 g/L which may severely prolong the initial lag phase (Michel-Savin et al., 1990b). In fed-batch fermentations using suspended cells and glucose as the carbon source, a butyrate concentration of 73 g/L, a productivity of 1.4 g/L h and a yield of 0.46 g/g have been reported (Song et al., 2010). With continuous systems, the productivity is generally higher but the butyrate and cell mass concentrations are lower. For example, at a

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dilution rate of 0.2 h^{-1} a productivity of 1.94 g/L h was achieved but the butyrate concentration was only 9.7 g/L and the butyrate yield 0.37 g/g glucose (Michel-Savin et al., 1990a).

In order to improve butyrate titers and productivities cell immobilization systems and continuous systems with cell recycle have been designed although their higher complexity might be discouraging for some applications. Using repeated fed-batch and fibrous beds for cell immobilization, butyrate concentrations of up to 86.9 g/L have been reported with glucose as carbon source (Jiang et al., 2011). Continuous systems with cell-recycle have achieved a very high productivity of 9.5 g/L h while maintaining a butyrate concentration of 29.7 g/L (Michel-Savin et al., 1990a).

For an economical and sustainable butyrate production, it is important to utilize low-cost and renewable medium components which preferentially do not compete with food production. The focus today has mainly been on the production of second generation biofuels and chemicals for which lignocellulose is the major raw material. Hydrolysates of lignocellulose and molasses are interesting raw materials for industrial production of many fermentation products. *C. tyrobutyricum* can utilize several carbon sources found in these materials including sucrose, glucose, fructose, xylose and arabinose, although some of them may not be co-utilized (Huang et al., 2011; Zhu et al., 2002). Currently there are few reports on butyrate production using lignocellulosic raw materials. Using a fibrous bed system and dilute acid treated corn fibre and corn steap liquor as the raw materials for fermentation, a butyrate concentration of about 26 g/L, a productivity of 2.91 g/L h and a butyrate yield of 0.47 g/g was reported (Zhu et al., 2002). *C. tyrobutyricum* RPT-4213 has been shown to produce butyrate on dilute acid pretreated wheat straw, corn fiber, corn stover, rice hull and switch grass, although the product concentrations were low (9 g/L) due to non-optimized processes (Liu et al., 2013). The major challenges with lignocelluosic materials are to achieve hydrolysates with high sugar concentrations and low levels of inhibitors.

Sweet sorghum is a potential renewable source for the production of biofuels. In a recent study comparing the production- and ecological sustainability of biofuel production from a selection of major crops, palm oil from south east Asia, sweet sorghum (*Sorghum bicolor* (L.) Moench) from China and sugar cane from Brazil were found to be the most sustainable (De Vries et al., 2010). Sweet sorghum has been shown to be a promising raw material for ethanol production displaying yields of 220 g ethanol per kg raw, dry stem, corresponding to about 2465 L ethanol per hectar (Cifuentes et al., 2014). Sweet sorghum contains about 50% soluble sugars (sucrose, glucose, fructose) and insoluble sugars (hemicellulose and cellulose) and has many attractive characteristics as a renewable energy source including short regeneration time (3–5 months), efficient utilization of soil and tolerance to high salinity, alkalinity, drought and flooding. The robustness of sweet sorghum enables the use of lands not suitable for cultivation of many food crops and hence there is little competition with food production. On the other hand, usage of sweet sorghum presents some difficulties, such as low storage stability and need for cooling during transportation and storage. Both are result of the high moisture and sugar content, which causes microbial contaminations. Microbial contamination can be prevented by drying of sweet sorghum stalks as previously demonstrated (Matsakas and Christakopoulos, 2013). Moreover, reduction of the water content results in decrease of stalks volume, which has a positive impact in the transportation and storage of the stalks. To the best of our knowledge, there are no reports on butyrate production using sweet sorghum.

Estimation on a fictive batch cultivation shows that in order to reach 70 g/L butyrate, with a yield of 0.4 g/g, the sugar concentration needs to be 175 g/L. Using fed-batch cultivation, the sugar concentration of the feed might need to be even higher. This is because a fed-batch cultivation may have to be started with a low sugar concentration to prevent sugar inhibition effects which in turn results in a dilution of the feed. To produce lignocellulosic hydrolysates with this sugar concentration, the fraction dry matter would need to be around 30–40% w/v. However, under such high dry matter conditions, cellulose fibers will bind the water more efficiently, causing mixing- and mass transfer problems (Lavenson et al., 2012). Enzymatic liquefaction can efficiently break the cellulose fibers so that the hydrolysate becomes more liquid-like. This could allow conventional stirred tank fermentations to be performed, and facilitate easy separation of the sugar containing liquid phase from the solids in the slurry (Matsakas and Christakopolous, 2013). Furthermore, in contrast to more severe treatments, like acid hydrolysis which generate comparably large amounts of growth inhibitors, enzymatic treatment generates a minimum of inhibitors.

To exploit the potential of *C. tyrobutyricum* to reach 70 g/L butyric acid with a fed-batch strategy, it is likely that the sweet sorghum juice needs to be supplemented with additional sugars to compensate for the dilution of the feed. Molasses, a byproduct of the sugar industry, is a well-established raw material for industrial fermentation. It contains mostly sucrose (~50% w/v) but also nitrous compounds, vitamins and other growth stimulating compounds (Olbrich, 1963). Expensive vitamin sources such as yeast extract could hence be replaced by molasses. Using a fibrous bed system and cane molasses, a butyrate titer of 26.2 g/L, a productivity of 4.13 g/L h, and a butyrate yield of 0.47 g/g was reported (Jiang et al., 2009).

The aim of the present work was to investigate sweet sorghum and beet molasses as a raw materials for butyrate production using *C. tyrobutyricum* as the production microbe.

2. Material and methods

2.1. Raw materials

Sweet sorghum stalks of Keller variety were offered by Prof. George Skarakis, Agricultural University of Athens. Stalks were dried as previously described and milled at 0.75 mm particles (Matsakas and Christakopolous, 2013). The molasses was prepared from beet and obtained from Nordic Sugar A/S, Denmark.

2.2. Enzymes, chemicals and strains

Commercially available Cellic® CTec2 (266 filter paper units/mL) was obtained from Novozymes (Novonordisk). The filter paper activity was measured according to Ghose 1987. *C. tyrobutyricum* (DSM 2637, ATCC25755) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). *Saccharomyces cerevisiae* invertase (Sigma I9274) was used for sucrose hydrolysis. Remaining chemicals and enzymes were obtained from Sigma–Aldrich.

2.3. Analysis of sugars and organic acids

Organic acids were determined by an HPLC system (PerkinElmer) equipped with a Series 200 refractive index detector and a BioRad Aminex HPX87-H column fitted with a BioRad Micro-Guard Cation H column. The column was kept at 65 °C and 5 mM H_2SO_4 was used as the mobile phase at a flow rate of 0.6 mL/min. Sugars were analyzed with the same system, except that a BioRad Aminex HPX87-P column fitted with a Micro-Guard Carb P column and a Micro-Guard De-Ashing column was used (H^+ and CO_3- form). Reference curves for the individual acids and sugars were prepared for the adequate concentration ranges. Concentrations were subsequently determined based on the peak

areas of the chromatograms in relation to the reference curves for respective substance.

2.4. Analysis of dry cell weight

The dry cell weight, expressed as grams of dry cells per liter, was determined gravimetrically by centrifuging 10 mL of the cultivation broth at 5000 × *g* for 10 min at 4 °C, followed by washing the cell pellet with two volumes (20 mL) of Milli-Q (MQ) H_2O. The pellet was subsequently resuspended in 5 mL MQ H_2O and dried until constant weight in a pre-weighed aluminum dish at 80 °C.

2.5. Analysis of free amino nitrogen (FAN)

The free amino nitrogen (FAN) was analyzed according to the EBC ninhydrin method (Lie, 1973). Briefly, samples and a glycine standard were diluted in Milli Q water and boiled for 16 min with the ninhydrin color reagent (4 g anhydrous Na_2HPO_4, 6 g KH_2PO_4, 0.5 g ninhydrin and 0.3 g fructose in 100 mL MQ H_2O) at pH 6.8. Samples were quickly cooled on ice to room temperature. Before measuring the absorbance at 575 nm, the samples were diluted with dilution reagent (2 g KIO_3 in 600 mL Milli Q water and 400 mL ethanol) and mixed until a stable color was obtained. The FAN in mg/L was calculated according to FAN = (A_{575} samples/A_{575} glycin standard) × 2 × dilution. Duplicate analysis were made on each sample.

2.6. Analysis of protein content

The protein content of the biomass and thin stillage reported as crude protein and Kjeldahl nitrogen, respectively, was determined according to the Kjeldahl method using block digestion and steam distillation (Suhre et al., 1982). The equipment included an InKjel P digestor and a behrotest® S1 distiller (Behr Labor-Technik, Germany).

Digestion was carried out by adding 20 mL of 98% H_2SO_4, antifoam and KT1 tablets (Thompson & Capper Ltd., United Kingdom) to 1.5–2.0 mL material for a total duration of 100 min at 100% power (of which 10 min for heating up the system). Digestion was followed by neutralization of the digested solution with 32% NaOH and distillation for 5 min. The distillation vapor was trapped in 50 mL of 4% H_3BO_4. Final titration was carried out with 0.1 M of HCl until pH 4.6. The commonly accepted nitrogen to protein conversion factor 6.25 was used to calculate the crude protein content (Mariotti et al., 2008). Duplicate measurements were made on each sample and data reported as mean ± standard deviation at 5% confidence level.

2.7. Analysis of cellulase activity

The cellulose (endoglucanase) activity was determined by incubating the samples with 1% w/v carboxymethyl cellulose as substrate, at 37 °C, for 16 h in 0.1 M citrate–phosphate buffer pH 5.0. The concentration of reducing sugars was determined using the dinitrosalicylic acid reagent (Miller, 1959). Glucose was used for the standard curve. One unit (U) of activity was defined as the amount of enzyme which released 1 μmol of glucose equivalents per minute under assay conditions. Blank reactions for the measurement of sugars that were already present in the culture medium were also included. Duplicate measurements were made on each sample and data reported as the mean in Fig. 6b.

2.8. Liquefaction of sweet sorghum stalks

Determination of the appropriate enzyme loads and dry matter fraction for liquefaction was conducted in 50 mL falcon tubes using 50 mM citrate buffer, pH 5.0, under the optimal conditions specified by the manufacturer for respective enzyme, with a holding time of 18 h. Sodium azide at a concentration of 0.02% was used to prevent microbial contaminations during the liquefaction trials. Enzyme loads of 1.5, 5, 10 and 30% of g dry matter were tested. The dry matter loadings tested were 25, 28.6, 30, 35 and 40% w/v. The number 28.6 comes from the fact that the enzyme load experiments were initially conducted using 25% w/w dry matter content, which was equal to 28.6% w/v dry matter content. Three independent liquefactions were conducted for each condition and data reported as mean ± standard deviation at 5% confidence level.

The hydrolysates used for the fermentation trials were prepared using cellic CTec 2 (Novozymes) and a dry matter loading of using 30% w/v. Briefley, 180 g of dried sorghum stalks was mixed with 50 mM citrate buffer (and enzyme preparation), at pH 5.0 to a final volume of 600 mL. Cellic CTec 2 (Novozymes) was used at a loading equal to 10% of dry matter (DM), equivalent to 26.5 filter paper units per gram dry matter. The enzyme preparation was diluted 10 times in 50 mM citrate buffer (pH 5.0) and filtred (0.45 μm) before use. The dry material was allowed to be completely soaked in the citrate buffer plus enzymes, at 50°C before addition of the citrate buffer to the final 600 mL. Liquefaction was performed at 50 °C for 18 h at 150 rpm. For fermentations with the solids the hydrolysate was autoclaved at 121 °C 20 min.

During the trials where the solids were removed, the hydrolysate was first filtered with a coarse filter. Subsequently, the flow through was centrifuged at 10,000 × *g*, for 10 min at room temperature (23 °C) and the supernatant was autoclaved as above.

When molasses was included in the feed, a mixture of 64% v/v non-autoclaved sorghum hydrolysate juice and 36% v/v molasses was autoclaved as above and used as feed for the fermentations.

2.9. Fermentation

The fermentations were carried out in 1 L stirred tank bioreactors (Applikon, the Netherlands) at a temperature of 37 °C, pH 6.0 and 250 rpm. The cultivation pH was controlled by automatic addition of 15% NH_4OH until 50 mL had been used, and then with 10 M NaOH for the remainder of the fermentation. The reason for the initial use of NH_4OH was that it improved the growth rate significantly compared to using only NaOH. However, using only NH_4OH as base caused toxic levels of NH_4OH to accumulate. It should be noted that NH_3 interferes with the EBC-ninhydrin method, and therefore, the FAN could not be determined in the cultivation broth. Anaerobic conditions were maintained by N_2 sparging 30 min before inoculation and during the fermentation, at a flow rate of 0.1 L/min. The inoculum was grown in an anaerobic box with an atmosphere of 90% N_2, 5% CO_2 and 5% H_2. The inoculum was grown at 37 °C in clostridial nutrient medium (Sigma 27546) and transferred to the bioreactor at a volume of 10% of the initial reactor volume.

The initial volume in the reactor was 200 mL. Fermentations performed without molasses had the following initial composition: 75 mg $FeSO_4 \cdot 7H_2O$, 1.125 mg KH_2PO_4, 450 mg $MgSO_4 \cdot 7H_2O$, 4 g yeast extract. The media was based on (Fayolle et al., 1990) and calculated for 750 mL end volume. Fermentations performed with molasses had the same composition except that the yeast extract was exchanged for 3.4% molasses (to obtain an equivalent of 20 g/L of sugars). Following inoculation *S. cerevisiae* invertase (Sigma I9274) was added at an activity of 1450 units per initial volume (7250 U/L), equivalent to 8.3 U/g DM when the hydrolysate was used as feedstock. One unit is defined as the conversion of 1 μmol of sucrose to glucose and fructose per minute at pH 4.6 and 25 °C. The sorghum hydrolysate with solids was manually fed to the reactor. The liquid feeds were fed with a peristaltic pump Alitea XV, using masterflex 96400-14 hose.

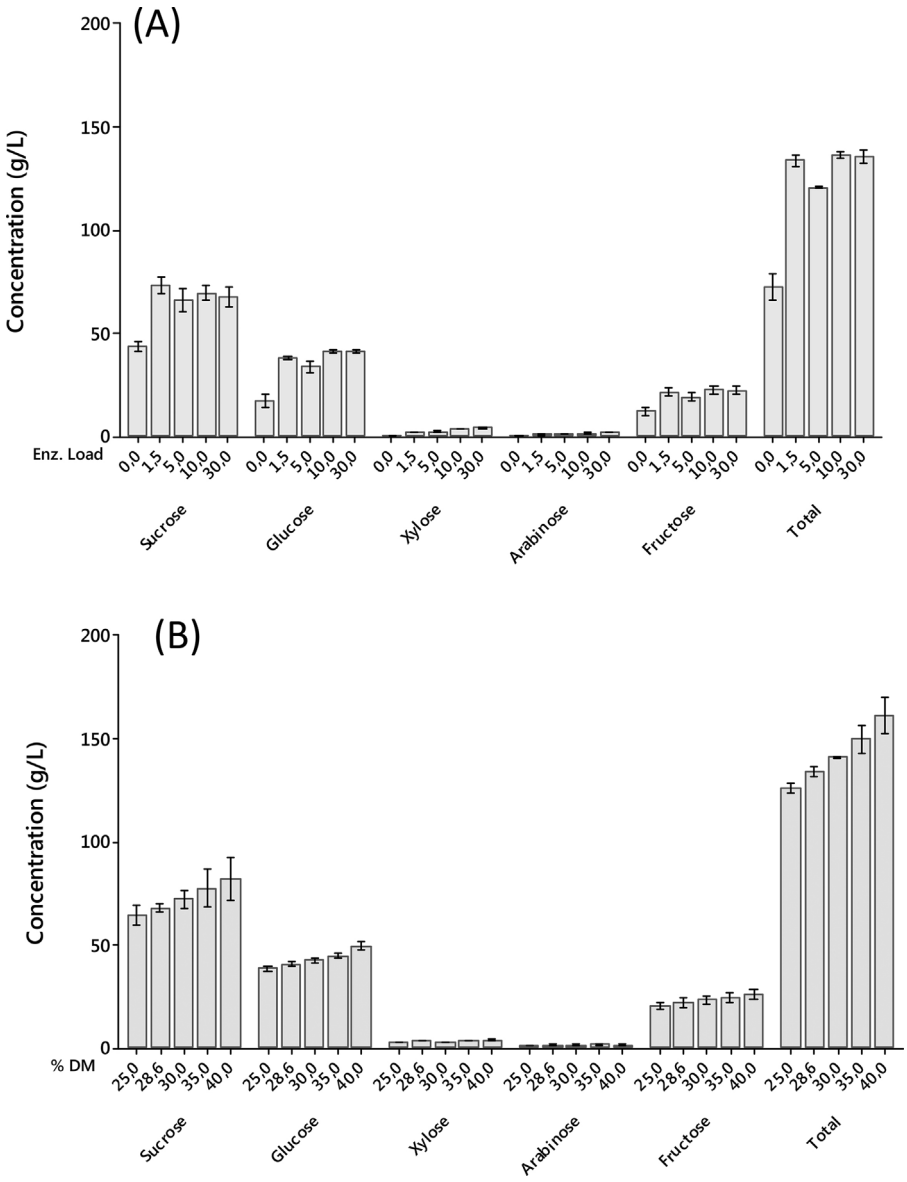

Fig. 1. Effects of different enzyme loads (A) and DM contents (B) on the sugar composition of the sorghum hydrolysate juice. Dried sorghum stalks at a DM content of 28.6% (w/v) was used during the screen with varying enzyme loads. An enzyme load of 10% of g DM (equivalent to 26.5 FPU/g DM) was used during the screen with varying DM contents. The screens were performed at 50 °C, pH 5.0 and 18 h. Three independant liquefactions were made for each condition. The mean and the standard error at a 5% confidence level is given.

2.10. Analysis of viscosity

The apparent viscosity of the non-hydrolyzed and hydrolyzed sweet sorghum stalks was measured using an Anton Paar Physica MCR rheometer apparatus (Anton Paar GmbH, Ashland, USA) as previously described (Karnaouri et al., 2014). The DM loading was 28.6% (w/v) during the measurements. One measurement was made for each condition.

3. Results and discussion

3.1. Evaluation of sweet sorghum liquefaction

The mixing- and mass transfer problems at high dry matter contents (>20%) make liquefaction a necessity. Liquefaction facilitates not only the reduction of the particle size and the slurry's viscosity, but also the enrichment of soluble sugars. The effect of the enzyme load (1.5, 5, 10 and 30% of g DM) on liquefaction of the stalks was investigated using a DM content of 28.6% (w/v) (Fig. 1a). Without enzymatic liquefaction, the total sugar concentration in the juice reached 72 ± 11.35 g/L and no xylose or arabinose was observed. When an enzyme load of 1.5% of g DM was used, a total sugar concentration of 133.8 ± 4.27 g/L was obtained. Increasing the enzyme load to 30% of the DM content did not increase the amount of extractable sugars, but the amount of xylose and arabinose appeared to increase in a dose dependant way. The appearance of xylose and arabinose suggests that there was xylanase activity in the Cellic Ctec2 preparation. In general, there was no increase in the sugar concentration with increasing enzyme load and most of the saccharification occurred already at an enzyme load of 1.5% of g DM. On the other hand, the change in viscosity was most pronounced with an enzyme load of up to 10% of the DM content (equivalent to 26.5 FPU/g DM) (Table 1). Hence an enzyme load of 10% of the DM content was used for the remainder of the liquefactions.

Subsequently, the effects of the DM content (25, 28.6, 30, 35 and 40% w/v) on liquefaction under constant enzyme load was

Table 1
Viscosity of the liquefied sorghum stalks (including solids) with varying enzyme loads.

Viscosity	(Pa.s)	38.05	14.53	12.20	9.21	8.75
Enzyme load	(% of DM)	0	1.5	5.0	10	30

evaluated (Fig. 1)b. As expected, the major carbohydrates of the hydrolysate juice were sucrose, glucose and fructose with some xylose and arabinose being present. Increasing the amounts of DM content resulted in a proportional increase in sugar content. The total sugar concentration in the juice increased from 125.6 ± 3.7 g/L to 161.3 ± 15.3 g/L when the DM content was increased from 25% w/v to 40% w/v. However, at 35% w/v DM content and particularly at 40% w/v DM content, the texture of the hydrolyzed material was similar to wet sawdust and was not practical to handle. Consequently, a DM content of 30% w/v was selected for the subsequent fermentations.

The hydrolysates used for the fermentations were hence generated by liquefying the sorghum stalks at 30% (w/v) DM using an enzyme load of 10% of g DM (equivalent to 25.6 filter paper units per gram DM) for 18 h at pH 5.0 and a temperature of 50 °C. Fresh hydrolysates were prepared prior to each fermentation and the mean composition of the hydrolysate juices is presented in Table 2.

3.2. Feed profile for the hydrolysate

Fed-batch cultivation has shown better product yields, productivities and product concentrations compared to batch cultivation, and therefore, a fed-batch strategy was adopted (Fayolle et al., 1990). However, the high solid content of the hydrolysate made it impossible to pump continuously with the available equipment and a semi fed-batch strategy was employed with a feeding profile according to Fig. 2. The feed profile was empirically developed for non-limiting sugar feeding, and to avoid excessive sugar inhibition. The highest specific growth rate of *C. tyrobutyricum* has been reported to occur at a glucose concentration between 10 and 60 g/L (Song et al., 2010). In this interval, the specific growth rate was shown to vary only between 0.38 and 0.48 h^{-1} and the maximum occurred at 23.6 g/L of glucose. For the initial trials, the feed profile in this study was developed with the aim of using as few additions as possible and sugar concentrations not exceeding 60 g/L, or falling below 5 g/L. Furthermore, it has been found that this strain of *C. tyrobutyricum* is not able to utilize sucrose even when present as the sole carbon source (Dwidar et al., 2013). By using a co-culture system with a *Bacillus* species capable of producing levansucrase, it was shown that sucrose could be hydrolyzed to glucose and fructose which subsequently were used by *C. tyrobutyricum* for butyrate production. However, co-culture systems are more complex than single culture systems and may put restrictions on the product yields as the carbon sources may be shared. In accordance with the literature it was observed, also in the present study that the cells were unable to co-utilize sucrose or to utilize sucrose when the other sugars were depleted (Fig. 3a and b). In order to have a controlled hydrolysis of sucrose during the fermentations, pure invertase was added at an activity of 1500 units per initial volume (7500 U/L). The amount of invertase was empirically determined and based on the observation that lower amounts than that specified tended to increase the residual sucrose concentrations. In order to avoid glucose inhibition of the cellulolytic enzyme, invertase was added directly to the reactor at the start of the fermentation.

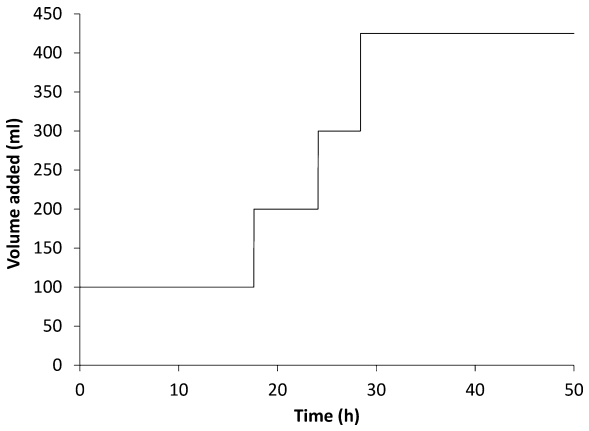

Fig. 2. General, pulsed feed profile used for runs with complete sorghum hydrolysate.

3.3. Fermentation with sorghum hydrolysate as the feedstock

When the hydrolysate (i.e., including the solids) was used as feedstock, the maximum butyrate concentration and butyrate yield were ($p = 0.05$), 37.2 ± 0.8 g/L and 0.39 ± 0.02 g/g consumed sugars, respectively, and the overall volumetric productivity was 0.86 ± 0.02 g/L h (Fig. 4a, Table 3). These data are consistent with those observed for cultures provided with a non-limiting feed of glucose although the acetate levels were slightly higher than commonly observed (Michel-Savin et al., 1990b). The high acetate levels are consistent with a high growth rate as the anabolic NADPH demand increase with increasing growth rate. Acetate production indicates regeneration of NADPH through NADP-dependent acetaldehyde dehydrogenase (E.C. 1.2.1.3). Glucose and fructose were utilized to completion, and about 2 g/L residual sucrose was found at the end of the fermentation (Fig. 4b). During the fermentation, the sugar concentrations were never limiting and never exceeded 35 g/L fermentable sugars. When the acetate concentration in the sorghum hydrolysate (6.5 g/L) was compensated for, the butyrate selectivity (conc. butyrate/(conc. butyrate + conc. acetate)) was 78% which is more common for batch cultivation (Fayolle et al.,

Table 2
Compositions of feed stocks used.

Feed stock	FAN[b] (mg/L)	Protein[c] (g/L)	Sucrose(g/L)	Glucose(g/L)	Xylose(g/L)	Arabinose(g/L)	Fructose(g/L)	Total sugars(g/L)
SHJ[a] (30% DM)	320 ± 26	18.8 ± 0.1	92.0 ± 7.6	50.9 ± 8.5	3.4 ± 0.9	1.6 ± 0.3	17.5 ± 5.9	165.3 ± 6.8
SM	NA	NA	100	50	–	–	20	170
Molasses	NA	NA	629					629
SHJ 64% + Mol 36%	587 ± 22	53.1 ± 0.1	286	29.4	1.0	–	10.5	326.9
SM 64% + Mol 36%	NA	NA	289	29	–	–	17	335

SHJ = sorghum hydrolysate juice; SM = sugar mix; Mol = molasses; DM = dry matter.

[a] The sugar concentrations of the sorghum hydrolysate juice used for the fermentations are reported as mean ± standard deviation at 5% confidence level, using 4 independent liquefactions.

[b] FAN (free amino nitrogen) is determined according to the EBC-ninhydrin method (Lie1972). Duplicate analysis of one hydrolysate sample and one feed (SHJ 64% + mol 36%) sample was done. Mean ± std.dev. at 5% confidence level is given.

[c] Protein content is reported as Kjeldahl protein using a conversion factor of 6.25. Duplicate analysis of one hydrolysate sample and one feed (SHJ 64% + Mol 36%) sample was done. Data reported as mean ± std. dev. at 5% confidence level.

Table 3
Summary of the process data.

Feed stock	Max butyrate concentration (g/L)	Butyrate yield[b](g/g)	Butyrate productivity (g/L h)	Max acetate(g/L)	Max dry cell weight(g/L)
Using pulsed feed profile					
SH (+invert.)[a]	37.24 ± 0.83	0.39 ± 0.02	0.86 ± 0.02	13.70 ± 0.70	–
SHJ (+invert.)	36.8	0.41	1.26	14.4	12.8
SM (no invert.)	21.2	0.46	0.51	4.26	3.3
SM (+ invert.)	47.2	0.44	0.72	8.5	6.6
Using constant feed profile and molasses					
SHJ 64%Mol 36%	58.8	0.52	1.92	11.46	15.7
SM 64%Mol 36%	50.6	0.48	1.6	3.41	14.7

SH = sorghum hydrolysate (i.e., including solids); SHJ = sorghum hydrolysate juice; SM = sugar mix; Mol = molasses; Invert. = invertase.
[a] Data based on two independant fermentations. Data reported as mean ± std. dev. at 5% confidence level.
[b] Yield based on g consumed sugars.

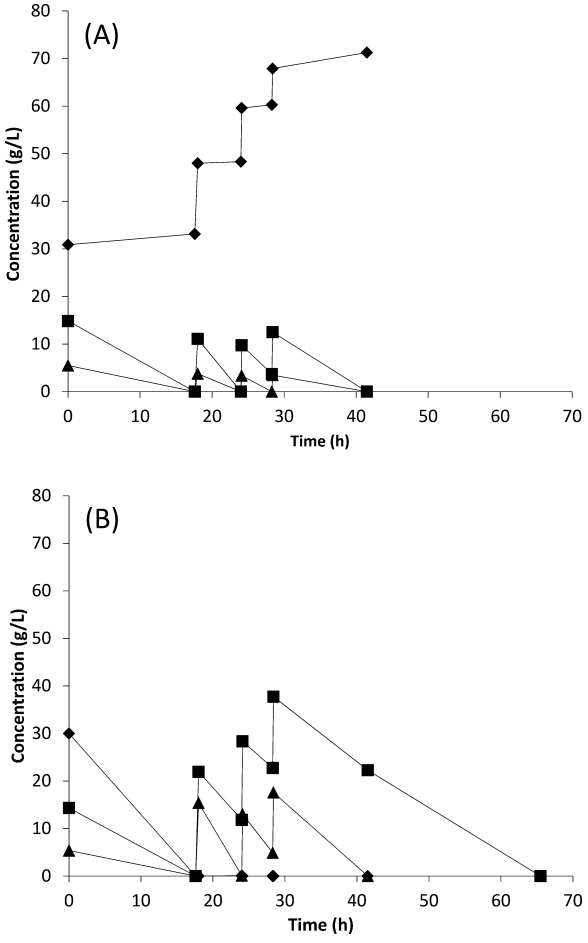

Fig. 3. Time course of sugar concentrations during fermentations supplemented with invertase (A) and not supplemented with invertase (B), using the pure sugar mix similar in composition as the sorghum hydrolysate juice. The pulsed feed was used. (♦) sucrose, (■) glucose, (▲) fructose.

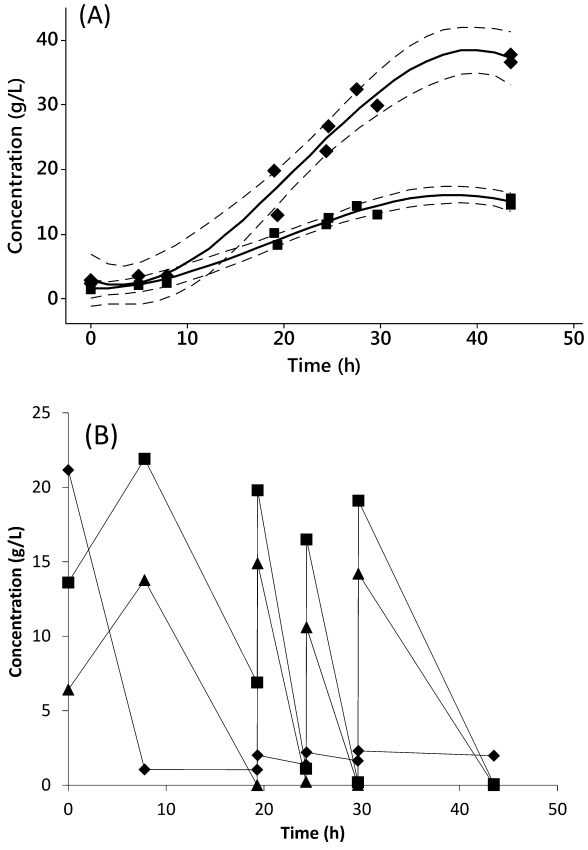

Fig. 4. Time course of butyrate and acetate concentrations (A) and sugar concentrations (B) for fermentation with the sorghum hydrolysate as the feed-stock, using the pulsed feed. For the butyrate and acetate concentrations, data points right before the hydrolysate additions are provided. For the sugar concentrations, data is given for one fermentation right before and after additions. In A: (♦) butrate, (■) acetate; in B: (♦) sucrose, (■) glucose, (▲) fructose. Two independent fermentations were performed and cubic regression curves were fitted to the data. (- - - -) represents a 95% confidence interval. R^2 for butyrate was 97.5% and for acetate 98.2%.

1990). In general, three fermentation phases can be observed for *C. tyrobutyricum* (Michel-Savin et al., 1990b). In the first phase, the growth phase, exponential growth is observed in parallel with simultaneous butyrate and acetate production. In the second phase, cell growth is reduced and acetate reutilization is observed. Finally, a third phase with low metabolic activity can be observed. The rather high acetate levels and the low acetate reutilization observed therefore suggest that the cells remained in the first phase for the most part of the fermentation. The low acetate re-consumption can be attributed to the fact that all sugars were consumed before, or at the beginning of the second phase and that the following reactions are considered:

$$\text{Glucose} \rightarrow \text{butyrate} + 2CO_2 + 2H_2$$

$$\text{Glucose} + 2H_2O \rightarrow 2\text{acetate} + 2CO_2 + 4H_2$$

$$\text{Glucose} + 2\text{acetate} \rightarrow 2\text{butyrate} + 2CO_2 + 2H_2O$$

Presence of solids in the hydrolysate prevented quantification of the cell mass concentration. The volumetric sugar consumption rate between 20 and 30 h using pure sugars was about 3.2 g/L h compared to 7 g/L h when using the sorghum hydrolysate. Assum-

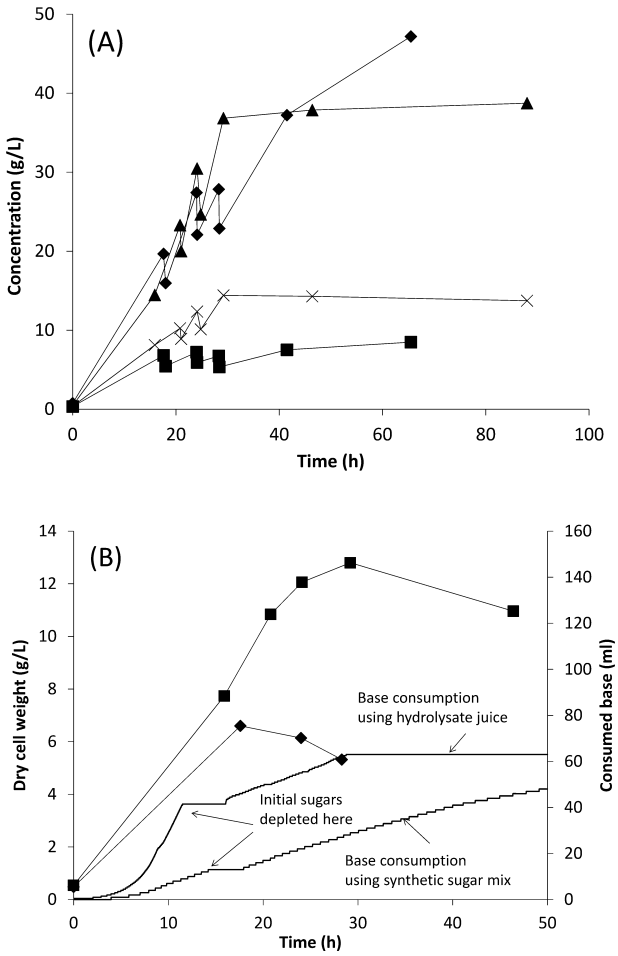

Fig. 5. Time course of butyrate and acetate concentrations (A) and cell mass concentration and base consumption (B) during fermentation with sorghum hydrolysate juice or pure sugarmix as feedstocks. The pulsed feed was used and the fermentations were supplemented with invertase. The total sugar concentration of the pure sugar mix was slightly higher (170 g/L) compared to the sorghum juice (160 g/L). In A using sorghum hydrolysate juice: (▲) butyrate, (X) acetate; using sugar mix: (♦) butyrate, (■) acetate; in B (♦) using using sugar mix, (■) using sorghum hydrolysate juice. Please note that the base consumption curves are only used to show when the initial sugars are depleted and do not reflect the exact volume base consumed.

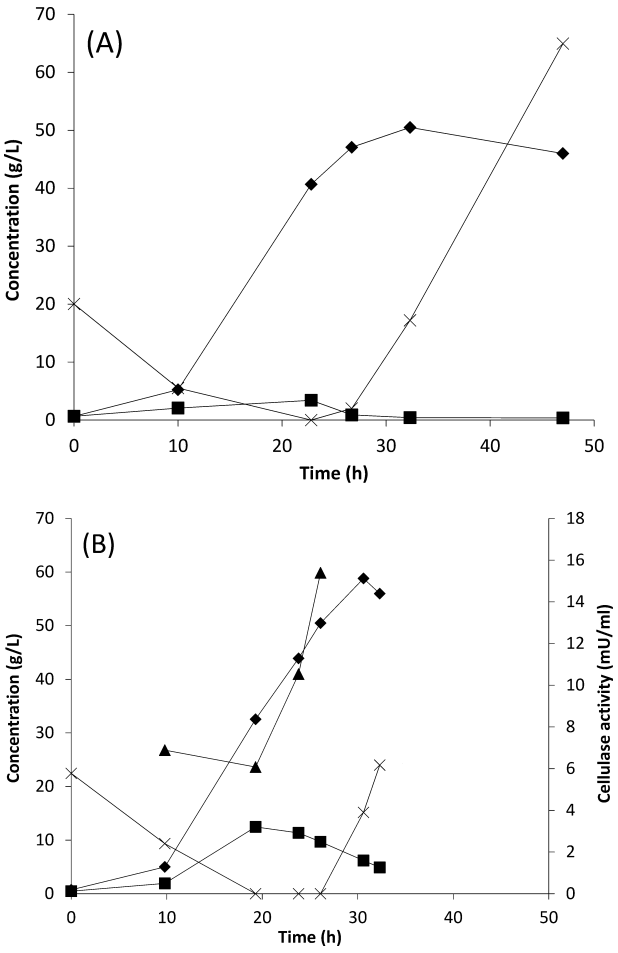

Fig. 6. Process kinetics for fed-batch fermentation with constant feed and molasses supplementation. The constant feed was started at 10 h. (A) Feed-stock was 64% v/v pure sugarmix (similar in composition as the sorghum hydrolysate juice) and 34% v/v molasses. (B) The feed stock was 64% v/v sorghum hydrolysate juice and 34% v/v molasses. The cellulase activity was also investigated and performed with carboxymethyl cellulose as substrate at 37 °C, pH 5.0 and 16 h. (♦) butrate, (■) acetate, (X) total sugars (fructose + glucose + sucrose), (▲) cellulase activity.

ing a similar specific sugar consumption rate during this time frame, the cell mass concentration using the sorghum hydrolysate would be about twice that of the fermentation using pure sugars.

3.4. Fermentation using the hydrolysate juice as feed-stock

In order to investigate the influence of the sorghum solids during fermentation, they were removed by filtration and centrifugation. Subsequently, the hydrolysate juice was used as substrate and the fermentations performed as previously described (Fig. 5a and b). In general, it was found that the fermentation kinetics were similar but faster without solids (compare Figs. 4 and 5a). The same high acetate/butyrate ratios and low acetate reconsumption were observed. Since the cells consumed the sugars more quickly without solids, the feed profile was adjusted so that the first addition occurred at 15.9 h instead of 19.4 h (when solids were included) and last addition was supplied at 25 h instead of 30 h (when solids were included). There was no sample taken directly after the addition of the sorghum juice at 15.9 h, and therefore, there is no jagged appearance at that point (Fig. 5)a. The negative influence of high solids content has also been found in other studies. For example, in one study, it was found that the H_2 production was negatively influenced by total solid contents above 18% using digestates from a methanogenic solid substrate anaerobic digester (Robledo-Narváez et al., 2013). The reason for the increased rates without solids could be related to the improved mass transfer and higher water activity.

The first addition of the sugar mix and the sorghum juice occured at 17.7 h and 15.9 h respectively. However for both cases the cells consumed all initial sugars before the first addition of respective raw material. This was in contrast to the fermentations using the complete sorghum hydrolysate with solids which never reached limiting sugar concentrations. Specifically, all sugars were consumed at 14.2 h with the pure sugar mix, and at 11.5 h with the sorghum juice as indicated by the base consumption curves where the sugar depletion can be seen as plateaus (Fig. 5)b. There was a direct correlation between the sugar depletion and the plateaus in the base consumption curves. Hence, there was a starvation period of about 3.5 h where the cells appear to have lost their ability to grow further on addition of pure sugar mix. However, when using the sorghum hydrolysate juice, there was a starvation period of 4.4 h and still the cells continued to grow when additional hydrolysate juice was fed to the reactor (Fig. 5)b.

Moreover, with the solids removed, it could be confirmed that the dry cell mass concentration was high reaching 12.8 g/L after 30 h (Fig. 5)b. This value was almost twice the cell mass concentration of 6.6 g/L obtained when the same fermentation was performed

with the synthetic sugar mix having a similar sugar composition as the sorghum hydrolysate juice (Fig. 5b). Cell mass concentrations reported for batch- and fed-batch cultivations using glucose as the carbon source and a similar basal salts medium (no peptones) as the present study range between 3 and 10.1 g/L (Jaros et al., 2013; Michel-Savin et al., 1990b). However, using reinforced clostridia medium, a dry cell concentration of 17 g/L has been reported for fed-batch cultivation (Song et al., 2010). The comparison therefore suggests that the growth promotive properties of the sorghum hydrolysate juice may be linked to its protein or amino acid content.

The protein concentration in the sorghum hydrolysate juice was determined to be 18.8 ± 0.1 g/L ($p = 0.05$) according to the Kjeldahl method (Table 2). The rather high protein concentration is in sharp contrast to the pure sugar mix and is likely to have stimulated growth. However, *C. tyrobutyricum* does not belong to the proteolytic clostridia and it is questionable whether this strain can digest proteins and/or peptides (Mead, 1971). On the other hand, free amino acids have been shown to be assimilated by *C. tyrobutyricum,* and could hence, be an important carbon and nitrogen source, particularly during growth (Chao et al., 2015). The free amino nitrogen (FAN) in the sorghum hydrolysate juice was determined to be 320 ± 26 mg/L by the EBC-ninhydrin method (Lie, 1973) (Table 2). This is a similar amount to that obtained with a standard 2% peptone solution (typical free α-amino nitrogen of peptone powder is 3% – http://www.organotechnie.com/doc_en/AI230.pdf).

The enzymatic hydrolysis was performed in 50 mM citrate and it might be argued that the citrate could act as an additional nutrient not present in the fermentations performed with pure sugars. However, this is not likely the reason for the high acetate levels as similar fermentations using liquefied corn cob as raw material did not generate high acetate levels (data not shown). Although the protein and FAN in the sorghum hydrolysate juice appear to be important factors for the high growth rate and acetate levels, other growth stimulating components of the juice cannot be excluded.

3.5. Conclusion from the pulsed fermentations using sorghum hydrolysate

With its growth stimulating properties, the sorghum hydrolysate was shown to be a very promising feed-stock for butyrate production. However, for fed-batch cultivation the sugar concentration of the hydrolysate was too low to reach the target of 55–70 g/L butyrate. In addition, because acid inhibition (butyrate plus acetate) starts at around 50 g/L, the high acetate levels reached during the fermentations also limited butyrate production. Hence, to improve the final butyrate titers, the sugar concentration of the feed would have to be increased and the acetate levels during the fermentations would have to be reduced in favor of butyrate production. Furthermore, the fermentations until this point had been performed with yeast extract which is an expensive component for industrial applications where molasses would be more appropriate. The next step was to improve the conditions for butyrate production using sorghum stalk hydrolysate as the major feedstock.

3.6. Process improvement

The targets of improvement were volumetric productivity, product concentration and reduced acetate levels (i.e., to improve the butyrate specificity). It has been shown that butyrate production follows a mixed-growth-associated pattern where the growth associated parameter is much larger (51.2 times for glucose) than the non-growth associated parameter, using the Luedeking–Piret model (Song et al., 2010). In order to improve the volumetric productivity, it was therefore important to make the cells grow as fast as possible. It can be noted however that the high growth rate also directs the carbon toward cell mass and a higher fraction of acetate, instead of butyrate. To reach a higher carbon to product efficiency, it could be argued that the production phase should be the stationary phase. However, the butyrate productivity during this phase is very low, and in order to compensate for the lower butyrate productivity during the stationary phase, an exceptionally high cell mass concentration would have to be used. More complex process systems, such as cell immobilization systems or continuous systems with cell-recycle could achieve that (Jiang et al., 2009; Du et al., 2014).

The maximum specific growth rate of *C. tyrobutyricum* has been reported to be at a glucose concentration of 20 g/L (Song et al., 2010) and so the start concentration of sugars for the present study was set to 20 g/L. The high acetate levels associated with high growth rates could be counteracted by providing limiting feeds of sugars which can force the cells to consume the acetate (Michel-Savin et al., 1990b). The strategy was therefore to balance the feed so that the cells would grow as much as possible and then starve them to promote consumption of acetate resulting in butyrate concentrations close to the inhibiting levels at 50–70 g/L.

The highest reported butyrate concentration for suspended cells is 73 g/L (Song et al., 2010). The minimum sugar concentration required to reach this butyrate concentration, assuming an initial volume of 220 mL, a base addition of 60 mL, a max sugar feed volume of 450 mL and a yield of 0.4 g butyrate per gram sugars, would be approximately 300 g/L. In order to improve the low sugar concentration in the sorghum stalk hydrolysates (~160 g/L), they were supplemented with molasses. To make a sugar feed with approximately 300 g/L of sugars, a mixture of 64% sorghum hydrolysate juice and 36% molasses were used. Molasses was also chosen as a source of vitamins so that the yeast extract could be omitted.

To ensure that the cells were not overfed during the initial phase, the cultivation was started as a batch cultivation with initial sugar concentration of 20 g/L. Subsequently, when the cells had entered exponential growth and consumed approximately 2/3 of the initial sugars, a constant sugar feed of 6.85 mL/h (equivalent to 2.25 g sugars/h) was started.

3.7. Fed-batch fermentation on the hydrolysate juice/molasses mixture

In general, the fermentation kinetics were improved by using a constant feed and molasses to increase the sugar concentration in the feed (Table 3, Fig. 6a and b). Compared to the synthetic sugar mix, the sorghum stalk hydrolysate juice promoted a higher dry cell mass concentration, butyrate titer, butyrate yield and overall volumetric productivity. In fact, to the best of the authors' knowledge, the butyrate titer and overall productivity obtained on the sorghum juice hydrolysate and molasses are the highest reported for a crop feed stock and suspended cells.

However, when using the hydrolysate juice, the acetate levels were again much higher compared to when using the synthetic sugar mix, (compare Fig. 6a and b). The acetate contribution from the hydrolysate during these trials was even lower compared to the pulsed feed experiments, and is hence not the reason for the high acetate levels. As mentioned previously, a high growth rate has been found to be associated with increased acetate levels (Du et al., 2014). The volumetric cell mass productivity was 0.9 g/L h using the sorghum liquid and molasses versus 0.7 g/L h when using the pure sugar mix in combination with molasses calculated from 10 h when the feeds started (Fig. 7). Hence, the same general conclusion can be drawn as when the hydrolysate alone was used as the feedstock; it appears as the sorghum hydrolysate juice carries growth stimulating components which keep the cells in the early part of the growth phase.

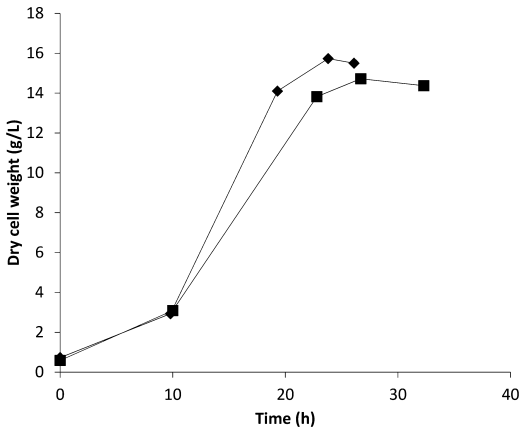

Fig. 7. Growth kinetics during fermentation with (♦) sorghum hydrolysate juice and (■) sugar mix. Constant feed started at 10 h.

3.8. Reutilization of the acetate

The strategy of balancing the feed of sugars to make the cells, consume acetate was successful. It can be clearly seen that when the total fermentable sugars become limiting, the acetate is readily consumed and higher butyrate specificity is achieved compared to the pulsed feed fermentations (Fig. 6a and b). This strategy could hence be particularly important when using sorghum hydrolysates which appear to promote high acetate levels. However, the conditions must be controlled so that the best combination of productivity, butyrate titers and yields can be reached.

3.9. High yields observed with the sorghum hydrolysate juice plus molasses

The butyrate yield for the fermentation utilizing, the sorghum hydrolysate juice and molasses (0.52 g/g) was higher than the theoretical maximum (0.489 g/g), suggesting that there are other carbon sources available in the feed in addition to sucrose, fructose and glucose. The feed containing pure sugars plus molasses also displayed a very high yield (0.48 g/g). Separate control fermentations were made with pure glucose and pure fructose, respectively, using 1% yeast extract and no molasses. From these control fermentations, a yield of 0.41 g/g glucose and 0.45 g/g fructose could be calculated which is consistent with the idea that there are additional carbon sources which the cells can assimilate in both the molasses and the sorghum hydrolysate juice.

Yields higher than the theoretical maximum (up to 0.54 g/g) was also found in another study where butyrate was produced on sugarcane bagasse and it was speculated that the reason for this was due to release of additional sugars from solid residues present in the feedstock (Wei et al., 2013).

Saccharolytic clostridia, including *C. tyrobutyricum* (Mead, 1971), is known to have cellolytic activites which are capable of breaking down plant material (Langston, 1958). Although *C. tyrobutyricum* is not mentioned specifically in the literature, several clostridia including *C. acetobutylicum* is capable of synthesizing a cellusome which can degrade carboxymethyl cellulose (a soluble form of cellulose), although at a low rate (Doi and Kosugi 2004; 541–551). In this study, it is possible that very small particles and larger sugar oligomers were present in the feed which could have been digested to sugar monomers and contributed to the high yield. To check for additional carbohydrates, the feed was subjected to an acid hydrolysis (treatment with concentrated HCl, 70 °C for 15 min). Following hydrolysis, no other sugar monomers than fructose and glucose were detected. However, the total sugar concentration (fructose and glucose) in the feed was increased from 326 g/L to 348 g/L indicating some remaining acid hydrolysable carbohydrates. The butyrate yield based on the sugar content of the hydrolyzed feed (sorghum hydrolysate and molasses) would be equivalent to 0.48 g/g.

3.10. Cellulase activity in the culture supernatant

To investigate if there was cellulase activity in the culture supernatant during the course of the fermentation, a cellulase assay was performed on the culture supernatant using carboxy methyl cellulose as the substrate (37 °C, 16 h, pH 5.0). Indeed some activity was found which more than doubled when the sugar concentration became limiting (Fig. 6)b. The increase in cellulase activity also closely coincided with the re-uptake of acetate. No activity from the Cellic CTec2 remained after autoclaving the hydrolysate, and therefore, the cellulase activity should originate from the cells. Taken together it could hence be speculated that the reason for the high butyrate yield is due to the presence of higher oligosaccharides which have not been accounted for and that these oligosaccharides eventually are degraded by cellulases expressed by the cells. Some acetate (4–6.5 g/L) was also present in the sorghum juice which can be converted to butyrate.

3.11. Amino acid utilization by the cells

The high protein content of the molasses (6.9% w/w specified by the manufacturer) could have contributed to the high butyrate yields, as free amino acids and peptides possibly could be used by *C. tyrobutyricum*. The FAN in the sorghum juice and in the feed was found to be 320 ± 26 mg/L and 587 ± 22 mg/L, respectively (Table 2). The FAN in the culture supernatant could not be determined because of the high NH_3 levels. These numbers appear to be too low to account for the high butyrate yield on their own unless proteins are degraded in the medium during the course of the fermentation.

Although *C. tyrobutyricum* does not belong to the proteolytic clostridia (Mead, 1971), genes of its genome has been assigned to amino acid permeases and intracellular proteases (Jiang et al., 2013) and it could be speculated that proteolytic activity and the assimilation of amino acids could be induced and/or enhanced during limiting feeds of sugars and in the presence of proteins.

Elucidating, the rather complex balance between additions of proteins, peptides, ammonia, and their consumption by the cells and possible proteolytic degradation of proteins and peptides to amino acids are beyond the scope of this paper. However, it is reasonable to assume that a certain amount of the amino acids are used by the cells which in turn could increase the butyrate yield on sugars.

4. Conclusion

Efficient enzymatic liquefaction of the sorghum stalks was achieved using up to 30% DM content, resulting in hydrolysate juices containing 165.3 ± 6.8 g/L of sucrose, glucose and fructose. The sorghum stalk hydrolysate showed great potential as a raw material for butyrate production using *C. tyrobutyricum*, resulting in high butyrate productivity and growth promotive qualities. In combination with molasses and fed-batch cultivation, the butyrate titers and productivities were the highest reported for freely suspended cells cultivated on crop feedstocks. Running fermentations with limiting feeds may be an important strategy to improve the efficiency of the fermentation as acetate is forced to be taken up and extracellular cellulase activity may be induced. The downside with the sorghum stalk hydrolysate was that invertase was required to hydrolyse sucrose into glucose and fructose, and that unusual high acetate levels were observed. On the other hand, with a controlled

feed, limiting the sugar supply at the end of the growth phase, the cells could be forced to reutilize the acetate ultimately resulting in an efficient process with good potential for industrial applications.

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