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Ethanol Production from Wet-Exploded Wheat Straw Hydrolysate by Thermophilic Anaerobic Bacterium *Thermoanaerobacter* BG1L1 in a Continuous Immobilized Reactor

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Abstract Thermophilic ethanol fermentation of wet-exploded wheat straw hydrolysate was investigated in a continuous immobilized reactor system. The experiments were carried out in a lab-scale fluidized bed reactor (FBR) at 70°C. Undetoxified wheat straw hydrolysate was used (3–12% dry matter), corresponding to sugar mixtures of glucose and xylose ranging from 12 to 41 g/l. The organism, thermophilic anaerobic bacterium *Thermoanaerobacter* BG1L1, exhibited significant resistance to high levels of acetic acid (up to 10 g/l) and other metabolic inhibitors present in the hydrolysate. Although the hydrolysate was not detoxified, ethanol yield in a range of 0.39–0.42 g/g was obtained. Overall, sugar efficiency to ethanol was 68–76%. The reactor was operated continuously for approximately 143 days, and no contamination was seen without the use of any agent for preventing bacterial infections. The tested microorganism has considerable potential to be a novel candidate for lignocellulose bioconversion into ethanol. The work reported here also demonstrates that the use of FBR configuration might be a viable approach for thermophilic anaerobic ethanol fermentation.

Keywords Ethanol · Wet-explosion · Thermophilic anaerobic bacteria · Wheat straw · Fluidized bed reactor · Lignocellulose

Introduction

The use of bioethanol in the transportation sector can reduce the current dependence of petroleum and reduce greenhouse gas emission, in particular, CO_2. These facts have

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increased the necessity of using alternative raw materials in addition to corn and sugar cane, which are nowadays the primary feedstock used for the production of fuel ethanol. Lignocellulosic biomass, like wood and agricultural residues (e.g., wheat straw, rice straw, corn stover, sugar cane bagasse), is an abundant renewable raw material for fuel ethanol production.

Wheat straw is one of the most important global feedstocks for ethanol production. Wheat straw is the major crop residue in Europe and the second largest agricultural residue in the world [1]. Potential global annual production of bioethanol from wheat straw (104 million cubic meters) could replace 75 million cubic meters of gasoline, or about 6.8% of the global gasoline consumption [1]. Wheat straw is the main agricultural waste product in Denmark and likely to be the lignocellulosic raw material used in a future Danish industrial bioethanol process. Since 2002, two pilot plants (IBUS and MaxiFuels) for production of ethanol from wheat straw have been inaugurated in Denmark.

Wheat straw, like other lignocellulosic materials, contains lignin, which tightly binds cellulose and hemicelluloses together, forming a complex, rather resistant structure. To make the cellulose and hemicellulose carbohydrates more susceptible to enzymatic hydrolysis and further microbial conversion, the lignocellulose must first be pretreated. However, the pretreatment conditions are severe, and various degradation products (e.g., acetic acid, furans, and phenols) are generated, which have an inhibitory effect on microbial growth and metabolism [2, 3]. To reduce the inhibitory effect of lignocellulosic hydrolysates, an additional detoxification step is needed [2, 3]. The inclusion of a detoxification process using a recombinant strain of *Escherichia coli* [4] has been found to increase the cost of ethanol production from acid-hydrolyzed willow by 22%. In this regard, the development of inhibitory-tolerant strains that are capable of tolerating toxicity of various hydrolysates is of large commercial interest.

Xylose is the major sugar present in the hemicellulose fraction of agricultural residues, such as wheat straw. Because the raw material cost is greater than one-third of the overall ethanol production cost [5], fermentation of xylose together with glucose is needed to improve the economics of any lignocellulosic-based bioethanol process.

Pentose-fermenting organisms are found among bacteria, yeast, and fungi, with the yeast *Pichia stipitis*, *Candida shehatae*, and *Pachysolen tannophilus* being the most promising naturally occurring xylose fermenting microorganisms [6]. However, the disadvantage with these organisms is their susceptibility to the inhibitors in the undetoxified hydrolysates and the difficulty in maintaining the oxygenation at optimal levels for optimum performance [7]. Metabolic engineering has been extensively used to develop recombinant strains of traditionally used ethanol producers like *Saccharomyces cerevisiae* and *Zymomonas mobilis*, and enteric bacteria such as *E. coli*, that will efficiently ferment mixed sugars glucose and xylose, and in some cases, arabinose [5, 6, 8]. However, cofermentation of a mixture of glucose with xylose and other sugars is commonly accomplished with either "glucose repression" or "xylose sparing" [2, 8] and instability of the plasmid carrying the xylose utilization genes in the presence of glucose over a longer time period [9]. In addition, recombinant organisms exhibit low tolerance to the inhibitors in undetoxified hydrolysates [5].

Thermophilic anaerobic bacteria are an alternative for pentose fermentation [10, 11]. The increased interest in these microorganisms arises from their ability to metabolize naturally both pentose and hexose sugars found in lignocellulosic hydrolysates. Moreover, there are a large number of potential advantages associated with the production of ethanol at higher temperatures, including high bioconversion rates, low risk of contamination, and energy

savings due to cost reduction via cooling, mixing, and distillation. However, these organisms are excluded from being seriously considered for bioethanol production primarily because of (1) low ethanol yields associated with the production of fermentation products other than ethanol, (2) limited data under conditions of practical interest including high substrate concentrations and real hydrolysate media, and (3) a lack of consensus in the literature on their tolerance to ethanol [10].

Because no one strain meets the requirement for efficient fermentation of lignocellulose, the development of industrial organisms capable of simultaneously fermenting the broad range of sugars present in lignocellulosic hydrolysates (particularly glucose and xylose) and tolerating the hydrolysate inhibitors is still a challenge [8]. In addition to the required industrial fermentation organism, continuous fermentation with high cell density (cell immobilization or cell recycle) could optimize the feasibility of the bioethanol production process by increasing ethanol productivity, ethanol concentrations and continuous ethanol removal [2, 11].

It was found that the wild-type strain *Thermoanaerobacter* BG1 could grow and produce ethanol from hemicellulose hydrolysate of wheat straw with the same ethanol yield as found for synthetic medium. To increase the ethanol yield of this organism, lactic acid production was eliminated by knocking out the lactate dehydrogenase gene, resulting in the strain *Thermoanaerobacter* BG1L1. It has been found that *Thermoanaerobacter* BG1L1 tolerates ethanol concentration of 8.3% (v/v), and thus, the ethanol tolerance of this strain is one of the highest found for thermophilic anaerobic bacteria [12]. Therefore, the objective of the present work was to investigate the cofermentation of glucose and xylose derived from undetoxified wet-exploded wheat straw by an immobilized cell culture of *Thermoanaerobacter* BG1L1.

Materials and Methods

Microorganism

The strain used in this study was *Thermoanaerobacter* BG1L1, which is a lactate dehydrogenase-deficient mutant of the thermophilic anaerobic bacterial strain BG1. The strains *Thermoanaerobacter* BG1 and BG1L1 have been deposited in the German Collection of Microorganisms and Cell Cultures as patented strains.

Media and Inoculum Preparation

Inoculum was prepared by growing cells in 100-ml vials containing 45 ml anaerobic synthetic medium (BA medium) [13], amended with 1 g/l yeast extract but without cystein. The medium was neutralized and flushed for 15 min with a mixture of N_2/CO_2 (4:1) to ensure anaerobic conditions before autoclaving at 140°C for 20 min. Prior to inoculation, the medium was reduced with a sterile anaerobic solution of sodium sulfide to a final concentration of 0.5 g/l. Xylose and vitamins were further added from filter-sterilized anaerobic solutions of D-xylose and vitamins DSMZ medium No141 (German Collection of Microorganisms and Cell Cultures) to initial concentrations of 5 g/l and 10 ml/l, respectively. The medium was inoculated with 10% (v/v) culture and incubated overnight at 70°C in the dark without shaking.

Wet-Explosion Pretreatment

Straw pellets were obtained from DONG Energy pellet plant in Køge, Denmark. Wheat straw pellets were pretreated by wet-explosion. Wheat straw pellets (200 g) were mixed with water (1 l) in a 3.5-l reactor and heated for 10 min. Hydrogen peroxide was added to reach a final wet-oxidation condition of 3% oxygen per dry matter content (DM, w/w) when the temperature was 170°C. The biomass was flashed as soon as all hydrogen peroxide had reacted with the biomass. The pretreated wheat straw out of the reactor was with a DM of 14% and consisted of hemicellulose, cellulose and lignin.

Hydrolysate Medium

Different pretreated wheat straw suspensions were prepared by the addition of a corresponding volume of water to give a concentration of 20–80% (wt/v), equivalent to wet-exploded wheat straw of 3–12% DM. The pH was raised to 5 with 10 M NaOH and the suspensions were autoclaved at 120°C for 20 min. After autoclavation the enzyme mixture of Celluclast and Novozyme 188 (3:1 v/v) (Novozymes A/S, Bagsvaerd, Denmark) was aseptically added to give 10 FPU /g-cellulose. Enzymatic hydrolysis was carried out in a shaker at 50°C for 2–4 days. After enzyme hydrolysis, the wet-exploded wheat straw hydrolysate was centrifuged at 4,000 rpm for 30 min at 4°C to remove the particulate matter. After hydrolysate centrifugation, the supernatant was collected and the pH was adjusted with 10 M NaOH to pH=7, followed by further supplements of yeast extract, minerals, trace metals, and vitamins as used in the anaerobic synthetic medium (BA). Finally, the hydrolysate medium (WEH) was flushed for 45 min with a gas mixture of N_2/CO_2 (4:1), and 250 mg/l Na_2S was added to ensure anaerobic conditions.

Reactor Set-up

A schematic diagram of the reactor set-up used in the present study is shown in Fig. 1. The reactor was a water-jacketed glass column with a working volume of 200 ml. The influent entered from the bottom of the reactor and the feeding was controlled by a peristaltic pump. Recirculation flow was achieved by using an identical peristaltic pump to ensure up-flow velocities in the reactor of 1 m/h. The pH was maintained at 7.0 by the addition of 1 or 2 M NaOH. Liquid samples were taken from a sampling port located on the top of the reactor, close to the reactor outlet. The experiments were performed at 70°C using external heating and the recirculation of hot water in the glass jacket.

Reactor Start-up and Operation

The reactor was loaded with 75 ml granular carrier material [14], and finally, the entire reactor system, including tubing and recirculation reservoir, was autoclaved at 120°C for 30 min. Before use, the reactor system was gassed for 15 min with N_2/CO_2 (4:1) to ensure anaerobic conditions and filled with BA medium with an initial xylose concentration of 10 g/l. The reactor was started up in batch mode by inoculation with 80 ml of cell suspension with an optical density (OD_{578}) of 0.9–1. The batch mode of operation was maintained for 24 h to allow cells to attach and to immobilize on the carrier matrix. After the batch run, the system was switched to continuous mode, applying a hydraulic retention time (HRT; the volume of the reactor divided by the influent flowrate) of 8 h and up-flow velocity of 1 m/h. To achieve operational stability, the reactor was run for 7 days under

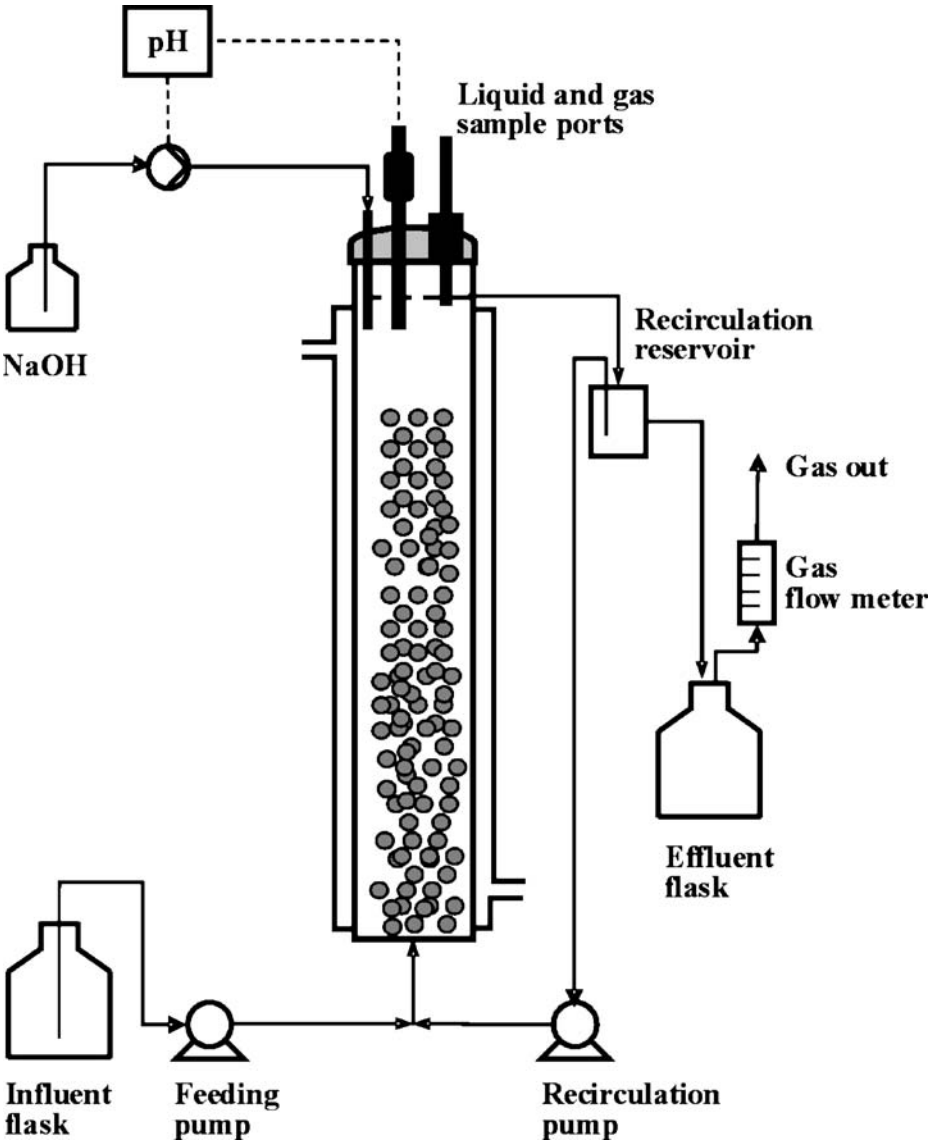

Fig. 1 A schematic diagram of the experimental set-up of the reactor

these operational conditions. After that, the fermentation started with 20% WEH as an influent at a HRT of 2 days. Unless otherwise stated, the HRT was 2 days. During the experiment, the concentration of WEH was gradually increased from 20 to 80% whenever steady state was achieved. The criteria for steady-state conditions were that all parameters must be held constant for at least five residence times. The reactor performance at different steady states was monitored by measuring the sugar and end-fermentation product concentrations. The values presented in the paper are the average of data taken at least over five residence times at steady state, and the standard deviation was less than 5%.

During the experiment, sterile syringes and needles were used to take the samples from the influent and effluent, and the samples were stored at −20°C until analysis.

Estimation of Ethanol Loss: Carbon and Redox Balances

The redox and carbon balances were used to determine the amount of carbon in the form of ethanol loss by stripping off to the gas phase. Lactic acid production at steady state conditions was less than 0.03 g/g; thus, it was excluded from carbon and redox balance calculations. For biomass production, biomass yield of 0.045 g/g was assumed [15, 16] with biomass composition $CH_{1.65}N_{0.23}O_{0.45}$ [17]. When growing the strain in batch culture, the carbon balance was almost closed (SD±2%), indicating that no other end products are formed than that included in redox and carbon balances.

Carbon recovery (Eq. 1) was determined as carbon in biomass and products formed divided by the total amount of sugar carbon consumed. For carbon recovery calculations, the amount of CO_2 produced was determined from the catabolic pathway of xylose and glucose, assuming that 1 mol CO_2 is produced for each mole of ethanol and acetic acid [15, 17–19]. Carbon balance can be written as:

$$CR[\%] = \frac{3(C_{\mathrm{EtOH}}/M_{\mathrm{EtOH}} + C_{\mathrm{Ace}}/M_{\mathrm{Ace}}) + C_{\mathrm{BM}}/M_{\mathrm{BM}}}{5C_{\mathrm{Xyl}}/M_{\mathrm{Xyl}} + 6C_{\mathrm{Glu}}/M_{\mathrm{Glu}}} \times 100 \tag{1}$$

where *Ci* is the concentration of compound *i*, i.e., substrate consumed or product produced (g/l) and *Mi* is the molecular weight of compound *i* (g/mol).

The redox balance was calculated based on fermentation products (ethanol and acetate) and biomass plus stoichiometrically associated production of H_2 accompanying the production of acetic acid [15, 17–19] as written:

$$RB[\%] = \frac{\gamma_{\mathrm{EtOH}}\phi_{\mathrm{EtOH}}C_{\mathrm{EtOH}}/M_{\mathrm{EtOH}} + \gamma_{\mathrm{Ace}}\phi_{\mathrm{Ace}}C_{\mathrm{Ace}}/M_{\mathrm{Ace}} + \gamma_{\mathrm{BM}}\phi_{\mathrm{BM}}C_{\mathrm{BM}}/M_{\mathrm{BM}} + 2N_{\mathrm{H2}}}{\gamma_{\mathrm{Xyl}}\phi_{\mathrm{Xyl}}C_{\mathrm{Xyl}}/M_{\mathrm{Xyl}} + \gamma_{\mathrm{Glu}}\phi_{\mathrm{Glu}}C_{\mathrm{Glu}}/M_{\mathrm{Glu}}} \times 100 \tag{2}$$

As the degree of reduction of xylose and glucose are equal, Eq. 2 can be reduced to Eq. 3:

$$RB[\%] = \left(\frac{Y_{\mathrm{EtOH}}\gamma_{\mathrm{EtOH}} + Y_{\mathrm{Ace}}\gamma_{\mathrm{Ace}} + Y_{\mathrm{Lac}}\gamma_{\mathrm{Lac}} + Y_{\mathrm{BM}}\gamma_{\mathrm{BM}} + 2Y_{\mathrm{H2}}}{\gamma_{\mathrm{Glu/Xyl}}}\right) \times 100 \tag{3}$$

where: γ is degree of reduction representing the electron content per C-mole for organic compounds or per mole for inorganic compounds; ϕ is the number of carbon atoms per mole of compound; N_{H2} is the concentration of H_2 (mol/l); and Y is product yield: for organic compounds, it is C-mole product per C-mole substrate and for H_2, it is H_2-mole per C-mole substrate.

The redox balance verified that missing product was with a high degree of reduction (5.7–5.9), confirming that the primary product missing is ethanol because the ethanol degree of reduction is 6. To validate the values of ethanol loss, ethanol evaporation rate was also determined experimentally in an experimental set-up identical to the one used for the WEH fermentations with medium containing 10 g/l xylose and 10 g/l ethanol. Measured ethanol concentrations in the effluent were used to estimate ethanol evaporation

rate (0.02 g l^{-1} h^{-1}). The ethanol concentrations, as directly measured by high-performance liquid chromatography (HPLC), were then corrected for loss of ethanol by applying this ethanol evaporation rate. The corrected ethanol concentrations fitted well with the ethanol values calculated from carbon and redox balances. Therefore, redox balance was used throughout the paper to determine ethanol loss, assuming that the missing percentage in the degree of reduction was due to ethanol loss, and to correct ethanol concentrations presented in the paper for loss of ethanol.

Analytical Methods

Glucose, xylose, and end-fermentation products (ethanol, acetate, and lactate) were quantified using an HPLC-RI equipped with an Aminex HPX-87H column (Biorad, Hercules, CA, USA) at 60°C with 4 mM H_2SO_4 as eluent and with a flowrate of 0.6 ml/min. In addition, the ethanol and acetate measurements were validated using an HP 5890 Series II gas chromatograph with flame-ionization detection and a silica capillary column (cross-linked polyethylene glycol-TPA; 30 m×0.53 mm). Prior to HPLC and gas chromatograph analysis, 1-ml samples were acidified with 10 μl 20% H_2SO_4 and 30 μl 17% phosphoric acid, respectively, and centrifuged at 10,000 rpm for 10 min, followed by filtration through a 0.45-μm membrane filter.

Test for Contamination

A 1-ml sample was taken from the reactor and chromosomal DNA was purified using the DNA purification kit from A&A Biotech (Gdynia, Poland). PCR reactions were setup using the Pfu polymerase (MBI Fermentas, St. Leon-Rot, Germany) and the primers B-all 27F (GAG TTT GAT CCT GGC TCA G) and B-all 1492R (ACG GCT ACC TTG TTA CGA CTT), which anneal to bacterial rDNA. The fragments were purified using the Qiaex II kit from Qiagen (Valencia, CA, USA), treated with PNK (MBI Fermentas), cloned into pBluescript SK+ (Stratagene, Cedar Creek, TX, USA) treated with CIAP (MBI Fermentas), and transformed into *E. coli* Top10 (Invitrogen, Carlsbad, CA, USA). Fifty clones were picked and the inserts were amplified using B-all 27F and B-all 1492R primers. The resulting fragments were digested with *Alu*I and *Mbo*I restriction enzymes (MBI Fermentas) and were run on a 3% agarose gel. Only one digestion pattern was found. Two fragments were sent for sequencing (MWG Biotech, Ebersberg, Germany) and were identified as strain *Thermoanaerobacter* BG1L1. PCR reactions were also run annealing, respectively, to regions upstream and downstream of the lactate dehydrogenase. Otherwise, the same reaction conditions as for the B-all primers, were used. The obtained fragments were cloned (as above); 26 were analyzed by restriction length polymorphism. Again, this resulted in only one pattern. Two fragments were sequenced.

Results

Thermophilic anaerobic ethanol fermentation of wet-exploded wheat straw hydrolysate was investigated using an immobilized culture of a lactate dehydrogenase-deficient mutant strain *Thermoanaerobacter* BG1L1. The fermentations were carried out in a lab-scale fluidized bed reactor (FBR) at 70°C. Whenever steady state was achieved, the concentration of WEH was increased gradually from 20 to 80% (wt/v), corresponding to sugar mixtures of glucose and xylose ranging from 12 to 41 g/l. Fermentation performance of tested strain

under steady state conditions was evaluated by sugar utilization, product formation (ethanol, acetate, and lactate) and ethanol yield. The results, including influent sugars and acetate concentrations, are summarized in Table 1. The experiment started with a HRT of 2 days to prevent substrate limitation due to short sugar residence time in the reactor and to enable the organism to adapt to the new environment.

With increasing of WEH concentration from 20 to 80%, the ethanol concentration in the effluent increased gradually from 4.6 to 14.42 g/l, yielding relatively high and stable ethanol yields in the range 0.39–0.42 g per gram sugars consumed, corresponding to 76–83% of the theoretically possible yield (Table 1). When the fermentation started with 80% WEH, the process continued at a HRT of 3 days to avoid kinetic limitation i.e., low reaction rate compared to substrate loading rate caused by higher concentration of inhibitors present at higher WEH concentrations. Increasing the HRT from 2 to 3 days was apparently effective with respect to utilized sugars, and 3% higher overall sugar conversion was seen compared to 60% WEH (Table 1).

Glucose utilization was higher than 90% for all tested WEH suspensions, whereas xylose conversion remained lower, between 72 and 80%. The lowest sugar conversions of 90 and 72%, respectively, for glucose and xylose were seen at 40% WEH. This could be attributed to a technical problem with the pH control system because the pH value was maintained around 6.5–6.7 instead of pH 7, and the reactor operated under these conditions (pH 6.5–6.7) with 40% WEH until the end of the experiment. The overall sugar conversion efficiency to ethanol for all these experiments was in a range of 68–76% (Table 1).

Acetate was the main by-product with a yield of 0.08–0.11 g/g (Table 1). High initial acetate concentrations (2.86–6.02 g/l) in the feed streams resulted in a rather high concentration of nearly 10 g/l acetate in the effluent at the highest WEH concentration tested (Table 1). In all fermentations, only trace amounts of lactate were produced (<0.03 g/g, data not shown) as expected, because the strain is a lactate dehydrogenase-deficient mutant. These data also show that deletion of the lactate dehydrogenase was stable over a long period of time.

During the experiment, which lasted for 143 days, the reactor was checked regularly for contamination by purifying chromosomal DNA from reactor samples, and no species other than *Thermoanaerobacter* BG1L1 were found. The deletion of the lactate dehydrogenase was also found to be stable, as shown by sequencing of the lactate dehydrogenase region.

Discussion

In a preliminary study on continuous cofermentation of glucose–xylose mixtures in a laboratory medium, the strain *Thermoanaerobacter* BG1L1 showed promising ethanol yields up to 0.45 g/g and an ethanol productivity of 1 g l^{-1} h^{-1} required for an economically feasible lignocellulose-based bioethanol process [20]. However, microorganisms producing high ethanol yields in laboratory media do not necessarily ferment lignocellulosic substrates efficiently because of the presence of a broad range of inhibitory compounds. Therefore, evaluation of fermentative performance of microbial candidates in lignocellulosic hydrolysates of industrial interest is crucial because the fuel ethanol production will be based on these substrates rather than laboratory media [21]. Thus, the fermentative performance of *Thermoanaerobacter* BG1L1 in cofermenting glucose and xylose present in undetoxified wet-exploded wheat straw hydrolysate was studied to verify the potential of this strain as a candidate for bioethanol production from lignocellulosic biomass. In this study, wet-exploded wheat straw hydrolysate was used as a lignocellulosic substrate

Table 1 Summary of ethanol production from undetoxified WEH by immobilized cells of the thermophilic anaerobic bacterium *Thermoanaerobacter* BG1L1 in a FBR operated at 70°C and pH 7.

HRT (days)	WEH (wt/v) %	Influent			Effluent				Sugar conversion			Yield[a]		Y_{EtOH}[b] %	CE[c] %	CR[d] %
		Glu (g/l)	Xyl (g/l)	Ace (g/l)	Glu (g/l)	Xyl (g/l)	Ace (g/l)	EtOH[e] (g/l)	Glu (%)	Xyl (%)	Total[f] (%)	Ace (g/g)	EtOH (g/g)			
2	20	8.77	3.04	2.86	0.37	0.60	3.76	4.60	95.8	80.2	91.8	0.08	0.42	83	76	103.0
2	40	16.22	8.02	3.51	1.62	2.23	5.39	8.38	90.0	72.2	84.1	0.09	0.41	80	68	99.9
2	60	22.17	11.18	4.84	1.45	2.68	7.91	11.57	93.4	76.0	87.6	0.10	0.40	78	68	99.4
3	80	27.24	14.08	6.02	1.11	2.76	9.98	14.42	95.9	80.4	90.6	0.11	0.39	76	68	97.7

[a] Yield on sugars consumed

[b] Ethanol yield given as percentage of theoretical possible yield of 0.51 g/g

[c] Conversion efficiency is calculated by dividing the ethanol yield based on the glucose and xylose concentrations present in the influent by theoretical possible yield of 0.51 g/g

[d] CR carbon recovery

[e] Ethanol corrected for loss of ethanol based on redox balance

[f] Total sugar consumed (i.e., glucose plus xylose)

because it is likely to be the lignocellulosic feedstock for fuel ethanol production in the MaxiFuels pilot plant, Denmark.

To counteract possible bacterial contamination, the wet-exploded wheat straw was sterilized using autoclavation (120°C for 20 min). Autoclavation of wet-oxidized wheat straw (121°C; for 10–20 min) has recently been reported to increase the concentration of phenol acids, formic acid, glycolic acid, and malic acid by 30–40% and of acetic acid by 75% [22]. Despite the facts that wet-exploded wheat straw was temperature-sterilized and the hydrolysate was undetoxified, strain *Thermoanaerobacter* BG1L1 was capable of fermenting WEH with relatively high ethanol yields of 0.39–0.42 g per gram of sugars consumed (equivalent to 76–83% of theoretical ethanol yield) (Table 1). The ethanol yields are comparable with those previously obtained in a defined BA medium with similar glucose–xylose concentrations [20]. These results reveal that inhibitors present in the hydrolysate did not significantly affect the ethanol yield. Strain resistance to hydrolysate toxicity could be attributed to long-term continuous strain adaptation to inhibitors present in the hydrolysate coupled with the use of high cell mass concentrations (cell immobilization). Employing high cell densities for fermentation of lignocellulosic hydrolysates has previously been shown to overcome hydrolysate toxicity [2]. Recent studies with immobilized thermophilic yeast have demonstrated that immobilization increases tolerance to substrates, ethanol, and high osmolarity and stabilizes the fermentation capacity of the system in a wide range of pH and temperature conditions [23].

The lowest sugar conversions of 90 and 72%, respectively, for glucose and xylose were seen at 40% WEH. This could be attributed to a technical problem with the pH control system because the pH value dropped to 6.5–6.7 instead of pH 7. The reactor operated under these conditions (pH 6.5–6.7) with 40% (DM) WEH until the end of the experiment with this WEH concentration. The low sugar conversion at 40% WEH compared to all other substrate concentrations constitutes an example of the influence of small pH deviations on the fermentation. This is consistent with previous reports that pH is a crucial variable parameter during the fermentation of lignocellulosic hydrolysates, especially at high organic acid concentrations, because the inhibitory effects of organic acids increase with decreasing pH due to more acids being in the more toxic undissociated form [3]. Although process unbalance was caused by the loss of pH control, the long-term reactor performance (143 days) was demonstrated with respect to sugar conversions, ethanol yields, contamination, and stable lactate dehydrogenase strain mutation.

Continuous fermentation has previously been tested for cellulosic substrates (e.g., glucose conversion) by thermophilic anaerobic bacteria [24, 25]; however, the present study is, to our best knowledge, the first one dealing with continuous cofermentation of glucose and xylose derived from lignocellulosic biomass using these microorganisms.

Very limited data have been published regarding cofermentation of glucose and xylose present in wheat straw hydrolysate. Immobilized cells of yeast *Pachysolen tannophiluc* gave significantly lower ethanol yield (0.32 g per gram of sugars consumed from 35 g/l reducing sugars) and sugar efficiency (63.4%) during continuous fermentation of wheat straw hydrolysate than these reported here [26]. Recently, comparable ethanol production yields for recombinant *E. coli* FBR5 (0.33–0.41 g per gram of sugars available) have been reported for fermentation of similar glucose–xylose mixtures (41–46 g/l) present in undetoxified alkaline peroxide-treated wheat straw [27] and undetoxified dilute acid-pretreated wheat straw [28].

The ethanol yields obtained in this study are considerably higher than those reported for fermentation of the undetoxified hemicellulose fraction of wheat straw by various pentose-utilizing yeasts and are comparable with the highest yield of 0.41 g per gram of sugars

available achieved by batch culture of *P. stipitis* from detoxified hydrolysate [29]. The ethanol yields and sugar utilization presented in this paper are also somewhat higher than those (0.24–0.36 g per gram of sugars consumed) obtained from both undetoxified and detoxified wheat straw acid hydrolysates by the thermophilic bacterium *Bacillus stearothermophilus* T-13 during continuous fermentation with cell recycle [30]. The results obtained are also comparable to those from simultaneous saccharification and fermentation of the cellulose fraction from wheat straw by *S. cerevisiae* (0.35–0.42 g per gram of glucose potential in pretreated material) [31–33] and by thermotolerant yeast strain of *Kluyveromycess* species (0.32–0.41 g per gram of glucose potential in pretreated material) [34, 35].

The experimental data reported here seem rather encouraging in view of the feasibility of the tested strain (*Thermoanaerobacter* BG1L1) as a novel candidate for fuel bioethanol fermentation from lignocellulose. The high ethanol yield, the high sugar conversion, and the resistance to hydrolysate toxicity seen in this study provide additional evidence supporting the previous suggestions based on demonstrated high ethanol tolerance by *Clostridium thermosaccharolyticum* and *Thermoanaerobacter ethanolicus*: that the practicality of thermophilic anaerobic bacteria for industrial bioethanol production should be reevaluated [36, 37]. This study has also demonstrated that the use of FBR technology might be a viable approach for ethanol production by thermophilic anaerobic bacteria. Work is already under way to test the strain's performance in other undetoxified hydrolysates, and future experiments are under consideration to ferment higher-input sugar concentrations (e.g., higher biomass concentrations) and to optimize the process parameters to improve ethanol productivity.

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