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Research article

Potential of thin stillage as a low-cost nutrient source for direct cellulose fermentation by *Clostridium thermocellum*

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Abstract: Utilization of thin stillage (TS), derived from grain-based ethanol production, was investigated as an alternative source for microbial growth nutrients during direct conversion of cellulose by *Clostridium thermocellum* DSM 1237. Fermentation end-products synthesized by *C. thermocellum* grown on media prepared with various concentrations (50–400 g/L) of TS were compared to those synthesized by *C. thermocellum* grown on reagent grade chemical (reference) medium. Cell-growth in TS media, monitored with the aid of quantitative polymerase chain reactions (qPCR) technique, showed prolonged growth with increasing TS concentration. Final fermentation end-product concentrations from TS media were comparable with those from the reference medium despite lower growth-rates. The volumetric H_2 production generated by *C. thermocellum* grown with medium containing a low concentration (50 g/L) of TS matched the volumetric H_2 production by *C. thermocellum* grown in the reference medium, while higher concentrations (200 g/L) of TS resulted in greater synthesis of ethanol. Supplementation of TS-media with Mg^{++} enhanced ethanol production, while hydrogen production remained unchanged. These results suggest that TS, an attractive source of low-cost nutrients, is capable of supporting the growth of *C. thermocellum* and that high concentrations of TS favor synthesis of ethanol over hydrogen from cellulose.

Keywords: Dark fermentation; thin stillage; hydrogen; ethanol; cellulose; *Clostridium thermocellum*

1. Introduction

Fuels and chemicals, such as hydrogen and ethanol, synthesized by bacteria that utilize lignocellulosic biomass as a carbon source represent a cleaner, more economic, and most importantly, a sustainable supply of energy [1,2]. Single-step conversion of lignocellulosic materials by cellulolytic bacteria offers major savings over multistep non-cellulolytic processes [3,4]. The projected cost for ethanol production through consolidated bioprocessing was less than one-fourth of that projected for simultaneous saccharification with co-fermentation [3]. In microbial conversion processes, growth nutrients introduce a major cost factor, which can account for about 30% of the overall process cost [5]. Minimization of medium cost can potentially contribute to establish cellulosic biofuels as a viable alternative to petroleum refinery by improving the overall process economy. An ideal fermentation medium for commercial-scale production should support good microbial growth, be low in cost, and be readily available. Alternative nutrient sources for microbial processes have been investigated by numerous studies [6–9]. Development of an alternative medium composed of corn steep liquor (CSL) to reduce nutrient cost for ethanol production was reported [6] and CSL with yeast extract has been evaluated as nutrient sources for lactic acid production [7].

In dry-grind ethanol facilities, processing of distiller's grains is an energy intensive operation that is responsible for over half of the thermal energy requirement. After distillation, the bottom stream passes through a centrifuge to separate the most watery part (moisture > 90%) called thin stillage (TS), and the volumetric TS to ethanol ratio of a typical dry-grind ethanol plant is about 2.5 [10]. TS is then concentrated by multi-effect evaporators from 6–7% to 25–30% solids, which consumes more than 25% of the total energy expended by the ethanol production plant [11].

Residuals from yeast fermentation in TS were characterized by Kim, et al. [12], who found that TS could be an excellent source of complex nitrogen, minerals, and amino acids essential for microbial growth, and also provided good buffering capacity (pH 4–7). Thermophilic anaerobic digestion of TS was evaluated for methane production and the amount of methane produced was able to displace up to 59% of the natural gas consumed by the ethanol plant with reduction of volatile fatty acids concentration in TS [13]. Utilization of residual salts in TS ($CaCl_2$, NaCl, K_2SO_4, $NaNO_3$, $Mg(OH)_2$, Na_2SO_4, and KOH) was attempted as an aid for protein extraction from oil seeds [14]. Xylose, arabinose, and glucose obtained from TS-hydrolyzate with 10–40 g/L of glucose were converted into ethanol by recombinant *Zymomonas mobilis* ZM4 (pZB5) [15]. Since, none of the aforementioned studies considered insoluble substrates, further investigation is warranted to explore the capability of TS as a nutrient source for direct bioconversion of cellulose.

Synthetic media used in the laboratory are generally formulated from reagent grade chemicals. An approximate estimation of one such composition (Table S1, supplementary materials) shows the total cost per liter of medium as $CAD 6.73, which is unrealistically high for a commercial process. Assuming the price of TS as 10% of the DDGS market price [16], a formulation with TS and reducing agent (L-cysteine) cuts down the medium cost by over 90% ($CAD 0.57). Interestingly, the contribution of TS to this reduced cost is less than 1% while the rest is due to the reducing agent, which could be minimized in scaled up process.

Hydrogen (H_2) and ethanol are synthesized via core metabolic pathways in *C. thermocellum*. These pathways compete for carbon (H_2 synthesis is linked to synthesis of acetate) and electrons, and changes in concentrations of these end-products reflect metabolic shifts in response to medium

components [17]. In this study, TS from commercial ethanol production was investigated as a potential alternative nutrient medium for *Clostridium thermocellum* DSM 1237 during direct fermentation of cellulosic substrates. We compared growth and end-product (H_2 and ethanol) synthesis patterns by *C. thermocellum* grown in TS medium versus a reference medium prepared with reagent grade (generally 99% pure) chemical ingredients containing yeast extract and vitamins.

2. Materials and Methods

2.1. Microorganism and growth medium

Clostridium thermocellum DSM 1237 (synonymous collection numbers include ATCC 27405, JCM 12338, and NCIB 10682), obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), was used throughout this study. After two subcultures, aliquots from the DSMZ culture were stored in glycerol in −80 ^{o}C and were revived before each experiment.

All chemicals used were reagent grade and were purchased from Sigma (Sigma-Aldrich Co., Oakville, ON, Canada). A modified version of the defined MJ nutrient medium [18] was prepared by mixing the basal medium (per liter: 1.5 g KH_2PO_4, 2.9 g K_2HPO_4, 10.0 g MOPS, 150 mg $CaCl_2{\cdot}2H_2O$, 1 g $MgCl_2{\cdot}6H_2O$ (g), 1.25 mg $FeSO_4{\cdot}6H_2O$, 2.1 g urea, 1.0 mg resazurin and 3.0 g sodium citrate, 0.2 mg biotin, 2.0 mg pyridoxamine-HCl, 0.4 mg p-aminobenzoic acid and 0.2 mg cyanocobalamin) with one gram of yeast extract (YE). The medium included 10 g/L of α-cellulose powder as the carbon source unless otherwise stated. The medium was dispensed and capped into 60 mL serum bottles for a final working volume of 10 mL. Cysteine-hydrochloric acid was added to 1 g/L final concentration after making bottles anaerobic by repeated gassing with nitrogen and degassing cycles as described previously [19]. A 100-fold concentrated solution of urea and a 100-fold concentrated solution of vitamins were prepared separately and filter-sterilized into pre-sterilized and anaerobic bottles. These solutions were added aseptically to each experimental bottle to the desired final concentrations before inoculation. For all media preparation with TS, water was replaced with equal parts of TS (e.g. to obtain 100 g/L TS medium, 100 ml water was replaced with 100 g of TS slurry).

2.2. The source of TS

The Husky Ethanol plant situated in Minnedosa, Manitoba, Canada (Husky Energy Inc.) generously provided all TS used throughout this study. Three batches of TS were obtained and were preserved in −20 °C freezer for the experiments for up to 90 days after collection.

2.3. Experimental setup

Serum bottles (160 mL) with 60 mL working volume were used for all batch experiments conducted at 60 °C water bath continuously shaking at 100 rpm. Inocula were always grown on α-cellulose and were directly transferred to TS medium during inoculation. Reduced inocula volume (~ 5%) was used to minimize carry-over nutrients to the experimental bottles. Nutrient components were eliminated from original medium containing TS in two phases of batch cultures. In first phase, yeast extract (YE) and vitamins were excluded and in the second phase, only buffering agents were

added to TS media. As carbon source, α-cellulose at 10 g/L concentration was added to all excluding cultures that were tested for production from TS medium only. All TS concentrations are expressed as wet weight (as obtained from Husky ethanol plant) dispensed per liter of medium.

2.3.1. TS without yeast extract and vitamins

TS, at concentrations of 50 to 400 g/L, were added to medium containing without YE or vitamins. Samples were collected at 20 and 40 hours post-inoculation (h pi). A lower range of TS concentration, 5 to 50 g/L, was also examined in a separate batch test.

2.3.2. TS with only buffering agents

All components of regular MJ medium except buffers ($KHPO_4$, K_2HPO_4, MOPS) and rezazurin (redox indicator) were added to all TS media. Cellulose (10 g/L) was added to all cultures and incubated for 40 hours (h).

2.3.3. Fermentation end-product profiles on TS medium

A batch experiment was conducted for 65 h with six sampling time-points. TS concentrations of 50 g/L and 200 g/L were added to buffer solutions with or without cellulose. Analyses of fermentation end-products (organic acids and ethanol) were performed according to the procedures described by Islam, et al. [20].

2.4. Composition of TS

A TS sample was collected in May 2011 when the Husky Energy ethanol plant was operating with a feedstock that consisted of 85% corn and 15% wheat. TS samples were placed in aluminum pans and dried at 50 °C in oven and ground into a course powder (~ 1 mm size) before shipping to the Forage Laboratory of Dairy One Cooperative Inc. (New York, USA) for compositional analysis. Table 1 displays the composition of TS on a % dry mass (DM) basis. The last column indicates the amount of each ingredient in culture medium per 100 g/L of TS added. This analysis revealed that crude protein constituted nearly one-third of TS and the rest of the DM was comprised of carbohydrates, fibre, fat, minerals, and ash. Water-soluble components of the TS included carbohydrates (soluble sugars, such as glucose, xylose, arabinose and their polysaccharides) and organic acids (acetic acid and lactic acid). Table 2 presents a comparative overview of minerals present in the reference medium and the TS medium at 100 g/L concentration indicating TS deficient in several macro and micro-nutrients such as magnesium, calcium, iron, zinc and copper at this concentration.

Table 1. Composition of thin stillage (TS) analyzed by Dairy One Cooperative Inc. (New York, USA). Raw TS samples were obtained from the Husky Energy ethanol plant (Minnedosa, MB, Canada). Moisture content of TS was 92.3% ± 0.37.

Components		% DM	g/L (per 100 g of TS added)
Crude protein (CP)		32.3	2.49
	Available protein	28.7	2.21
	Soluble protein	9.4	0.72
Lignin		5.6	0.43
Cellulose		3.4	0.26
Hemicellulose		10.5	0.81
Sugar, starch, pectin, and fermentation acids		35.7	2.75
Water soluble carbohydrates		8.6	0.66
Crude fat		16.5	1.27
Ash		6.2	0.40

Table 2. Minerals in TS medium compared with the reagent grade chemical growth medium.

Minerals	TS medium—100 g/L	Chemical medium	
	(mg/L)	(mg/L)	µg/g of YE [21]
Mg^{2+}	50.31 ± 5.0	119.57	*1.27*
Ca^{2+}	7.0 ± 4.8	40.89	-
K^{+}	175.82 ± 42.5 (+1732.37)[a]	1732.37	-
P^{3-}	152.46 ± 31.8 (+856.94)[b]	856.94	-
S^{2-}	37.73 ± 10.7 (+182.6)[c]	182.6 (+1532.5)[d]	-
Na^{+}	58.49 ± 15.25	249.93	
Fe^{+}	1.13 ± 0.2	0.27	*150*
Zn^{2+}	0.55 ± 0.07	-	*74*
Mn^{2+}	0.32 ± 0.17	-	-
Cu^{2+}	0.06 ± 0.01	-	*71*
Ni^{2+}	-	-	*18.2*
Mo^{2+}	0.02	-	-

[a] and [b] potassium and phosphorus in the phosphate buffer; [c] sulphur in the reducing agent; [d] sulphur in MOPS

2.5. Monitoring growth of C. thermocellum on TS

2.5.1. Preparation of cell-suspension

C. thermocellum cultures were grown on 5 g/L cellobiose in balch tubes with 10 mL work volume and growth of cells was monitored with a spectrophotometer (Biochrom, Novaspec II) at 600 nm. When growth reached at an optical density (OD_{600}) of approximately 0.85, cultures were centrifuged (14,000 x g, 10 min) and the supernatant containing all solubles was removed. Pellets were washed with phosphate-buffered saline (PBS) contained 10 mM phosphate buffer and 150 mM NaCl at a pH of 7.4 and then re-suspended in PBS again for flow-cytometry work.

2.5.2. Cell count with flow-cytometry

Serial ten-fold dilutions of PBS cell suspension were prepared in rows of sterile 96-well plates. Ten (10) μL of sample was diluted in 90 μL sterile PBS, vigorously stirred, and mixed by pipetting, and then 10 μL was transferred to next well, etc. for up to 7 wells, depending on initial sample OD_{600}. The dilutions were stained with 100 μL SYTO-BC (fluorescent green) stain (10 μL diluted in 9.99 mL PBS to stain an entire plate).

For cell counts, approximately 3600 fluorescent green-yellow microparticles (ie. 10 μL of a tenfold dilution in PBS of microparticles with a known concentration of 3.6×10^6/mL) were added to each well. Plates were loaded into a FACS Array bioanalyzer (Becton-Dickinson, San Jose, USA) with standard sheath fluid, and the flow rate was set to 2 mL/s for 50 second (s) or 10,000 total events (laser signals captured). Voltages were set at the minimum for forward scatter, 350 for side scatter, 450 for yellow and 380 for green, with threshold set on green fluorescence to exclude unstained particles. Raw counts of cells and visualizations of cell populations were generated using FlowJo software (TreeStar Inc.).

2.5.3. Calibration curve with quantitative polymerase chain reaction (qPCR)

An identical stock of *C. thermocellum* cell suspension in PBS buffer was used to develop a qPCR calibration curve. Eight standards (10^{-1} to 10^{-8}) were prepared by ten-fold serial dilutions (PBS as diluent) starting from the original stock suspension. Pellets were obtained after centrifuge (14000 x g, 10 min) and removal of PBS supernatant. Genomic DNA, extracted and purified with the InstaGene matrix (#732-6030EDU, BioRad Laboratories Inc., Bio-Rad, Hercules, CA, USA), was used as template for qPCR. The DNA concentration in each standard was determined spectrophotometrically. The same kit was used to prepare DNA templates from experimental samples. The primer set was designed based on the chaperonin60 (*cpn60*) gene of *C. thermocellum*. Duplicate qPCR reactions for the standard curve, test samples, and no-template controls were prepared with the SsoFast EvaGreen® supermix (BioRad) with 2x PCR mix as presented in Table 3.

Table 3. qPCR reaction composition and primer sequences for the *cpn60* gene of *C. thermocellum*.

Total reaction volume = 25 μL		Primer sequence (amplicon length = 164)
Components	μL/tube	
Master mix	12.5	
Forward primer	1	5'-TCCAGGACATTCTCCCATTGCTGG-3'
Reverse primer	1	5'-TCTGTCACCAAAGCCAGGTGC-3'
Water	8.5	
DNA template	2	

Real-time qPCR was performed with the BioRad MiniOpticon System (48-well) equipped with CFX Manager software control. Real-time PCR was initiated with a denaturation step (95 °C for 3 min) followed by amplification of target DNA in 40 cycles. Each cycle consisted of denaturation (10 s at 95 °C), annealing (10 s at 55 °C) and extension (30 s at 70 °C). The amplification plot (S2) for standards and samples along with the calibration curve (S3) are included in the supplementary materials section.

3. Results

Growth nutrients were eliminated in two steps from the reagent grade chemical medium to observe if TS was able to replace them, partially or completely. In the first step, only yeast extract and vitamins were removed, and in the second step all other nutrients were removed, leaving only buffering and reducing agents, to which various amounts to TS were added as the sole nutrient source. A batch-culture time-profile was conducted with selected concentrations of TS determined by preliminary screening. Ethanol production was monitored in all cases, since it is a major fermentation end-product that competes H_2 synthesis for electrons. Background ethanol, acetate, and lactate (yeast metabolites) in TS media, and all carry-overs from the inocula, were subtracted from the total product concentrations measured to establish the end-product profiles generated by *C. thermocellum*.

3.1. TS media without YE and vitamins

Hydrogen production from cultures containing 50 to 400 g/L TS were compared to H_2 as well as ethanol production from the reference medium (control cultures) with equal amounts of added cellulose. In Figure 1, cultures with 50 g/L TS were found to produce the highest amount of H_2 and ethanol among all tested concentrations of TS. The H_2 and ethanol concentrations produced by *C. thermocellum* cultured with 50 g/L TS were very close to those produced by *C. thermocellum* grown with the reference medium. Average H_2 concentrations from cultures containing 50 g/L TS represented around 85–89% of H_2 from controls at 20 and 40 h pi, respectively. Ethanol concentrations produced from 50 g/L TS were similar to control cultures at both 20 and 40 h pi. Values of pH in all test cultures remained above 7.0, suggesting a negligible impact of pH on the ratio of fermentation end-products. Since the production dropped with increasing TS concentrations, it was assumed that one or more components in TS were inhibitory to growth of *C. thermocellum*.

Based on the initial experimental trials, which indicated that the lowest concentration of TS (50 g/L) resulted in H_2 and ethanol concentrations that were comparable to the control, a second experiment with a lower range of TS concentration (5 to 50 g/L) was examined (Figure 2). The results showed that all cultures produced significantly lower H_2 and ethanol concentrations compared with those from 50 g/L TS after 40 h pi. As H_2 synthesis mirrors cell growth, it was inferred that medium containing TS below 50 g/L contained nutrients in concentrations that were insufficient to support growth.

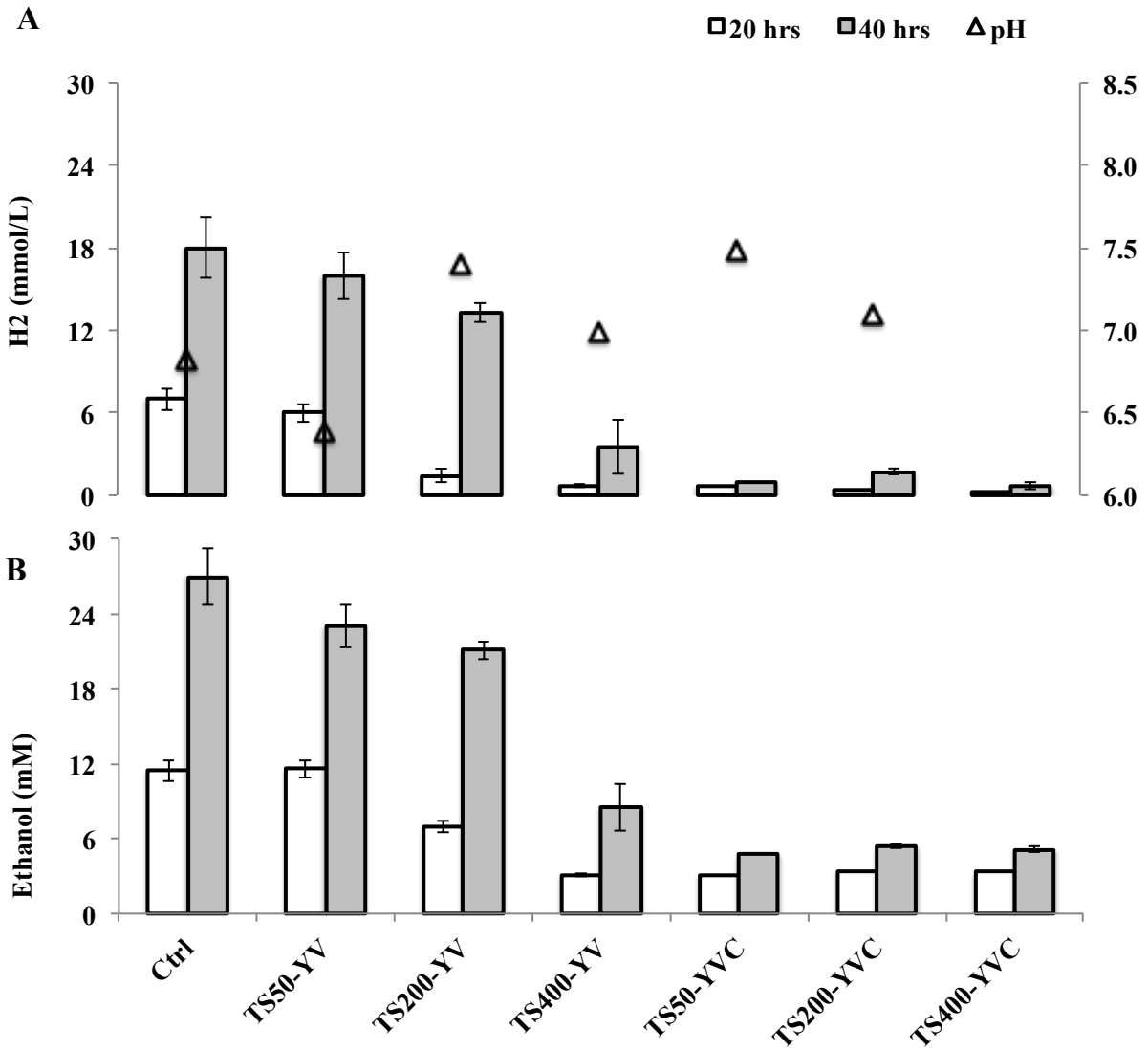

Figure 1. A) Hydrogen and B) ethanol production by *C. thermocellum* cultures grown on TS media with no added vitamins (V) and yeast extract (Y). Ctrl, control culture (*C. thermocellum* cultured with reference medium) containing 10 g/L cellulose and defined ingredients; TS (50/200/400)-YV, culture containing 50, 200 or 400 g/L TS with no added Y and V; TS (50/200/400)-YVC, culture containing 50, 200 or 400 g/L TS with no added Y, V and cellulose.

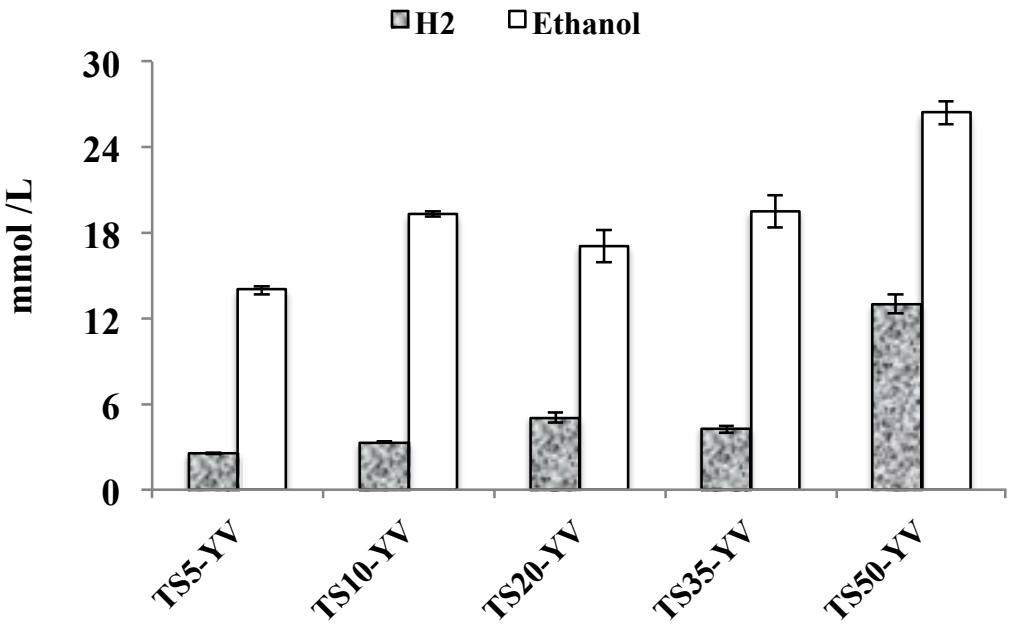

Figure 2. Gas production by *C. thermocellum* 1237 cultures grown on low thin stillage (TS) concentrations. TS were added at 5, 10, 20, 35 and 50 g/L to vitamins (V) and yeast extract (Y) free media.

3.2. TS media on buffers

Fermentation end-products after 24 h pi are shown in Figure 3, when all nutrients except buffering agents (phosphates and MOPS) were excluded from media containing 50, 100, 200 or 400 g/L of TS. Relative utilization of added cellulose by *C. thermocellum* was also verified for each concentration by culturing on corresponding TS medium with no added substrate. Overall, more total products were produced compared with previous batch cultures where only vitamins and yeast extract were excluded from TS media. Figure 3A shows that H_2 concentrations dropped significantly after TS addition exceed 50 g/L, while ethanol concentrations remained unchanged. Only a small amount (< 2 mM) of lactate was detected in TS medium. Also formate production was insignificant at all TS concentrations tested.

A small amount (3.4% of DM) of cellulose is present in TS (Table 1), which represents substrates fermentable by *C. thermocellum* and corresponds to about 0.8 mM of glucose equivalents when 50 g/L of TS is added to the medium. It is not surprising, therefore, that production from cultures with no added cellulose became significant with increasing amount of TS in medium. Production from cultures with 400 g/L TS, without added cellulose, represent up to 83% and 72% of the H_2 and ethanol, respectively, produced by cultures containing same amounts of TS plus cellulose. Utilization of added cellulose seems to be the highest in cultures with 50 g/L TS, since total production from cultures without added cellulose (50TS) were comparatively lower than those containing other concentrations of TS.

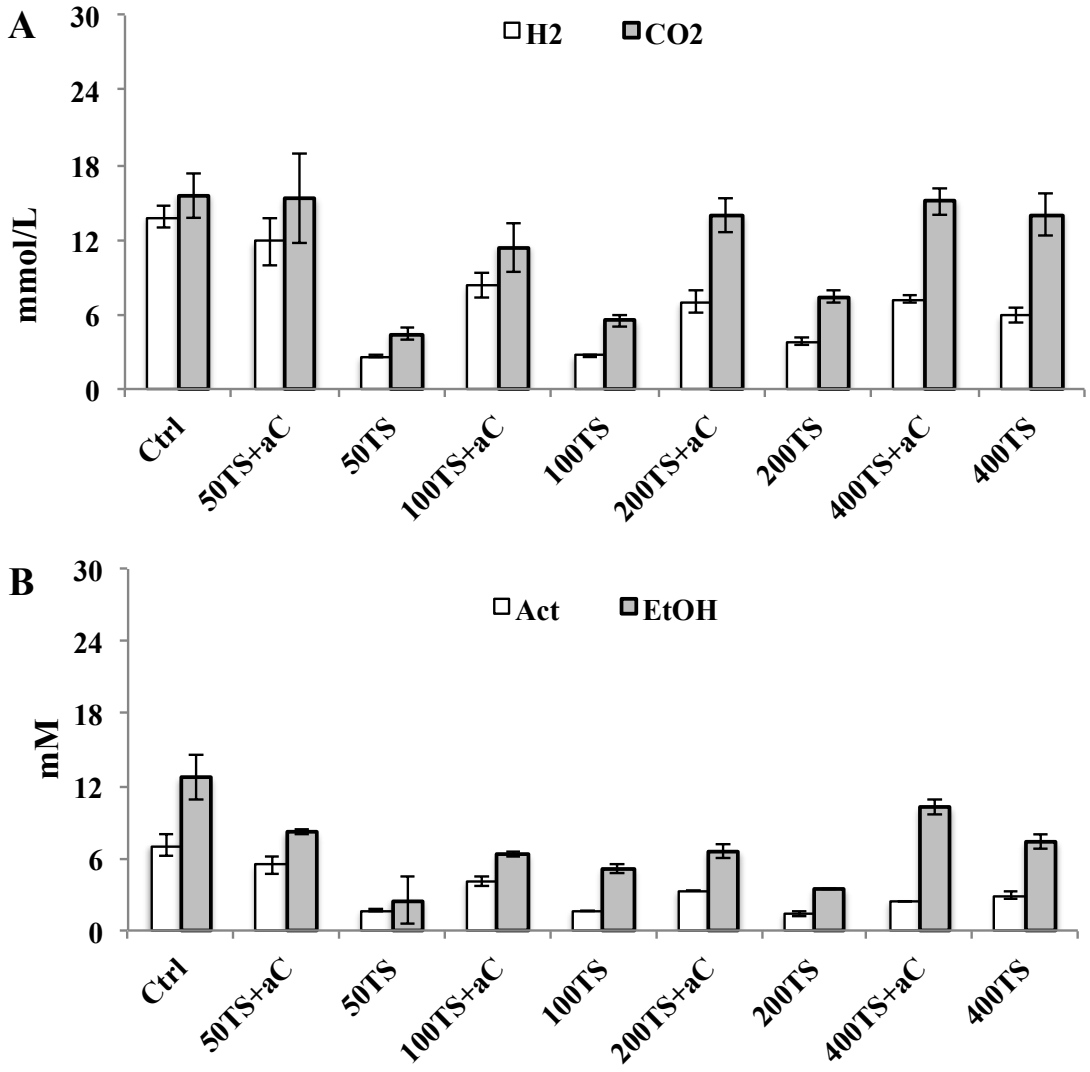

Figure 3. Relative production of A) gaseous and B) liquid end-products by C. thermocellum 1237 grown on TS media with and without added cellulose (aC). TS (50/100/200/400), cultures with TS but no cellulose; TS (50/100/200/400) + aC, cultures with 50, 100, 200 or 400 g/L TS plus 5g/L g/L-cellulose; Ctrl, control culture with regular medium and cellulose.

3.3. Time profiles of production on TS medium

Batch fermentation were carried out with 50 and 200 g/L TS (with and without added cellulose) to observe time profiles of H_2 and ethanol production by *C. thermocellum* (Figure 4). Final pH values remained above 6.5, so the culture pH drops did not affect product profiles. For first 25 h of incubation, carry-overs or background carbon in TS accounted for up to 50% of H_2 produced in cultures with 200 g/L TS. After that, production from TS only cultures plateaued, and the added cellulose was consumed in those cultures with cellulose. Cultures with 50 g/L TS produced H_2 at rates similar to the control medium (0.54 mmol/L.h). Despite a comparatively slower initial production rate from cultures

with 200 g/L TS, cumulative H_2 concentrations were similar by the end of the fermentation reactions. Ethanol was produced at similar rates in all cultures, except in 50 g/L TS, where it plateaued earlier resulting in 35% less total accumulation compared with 200 g/L TS cultures.

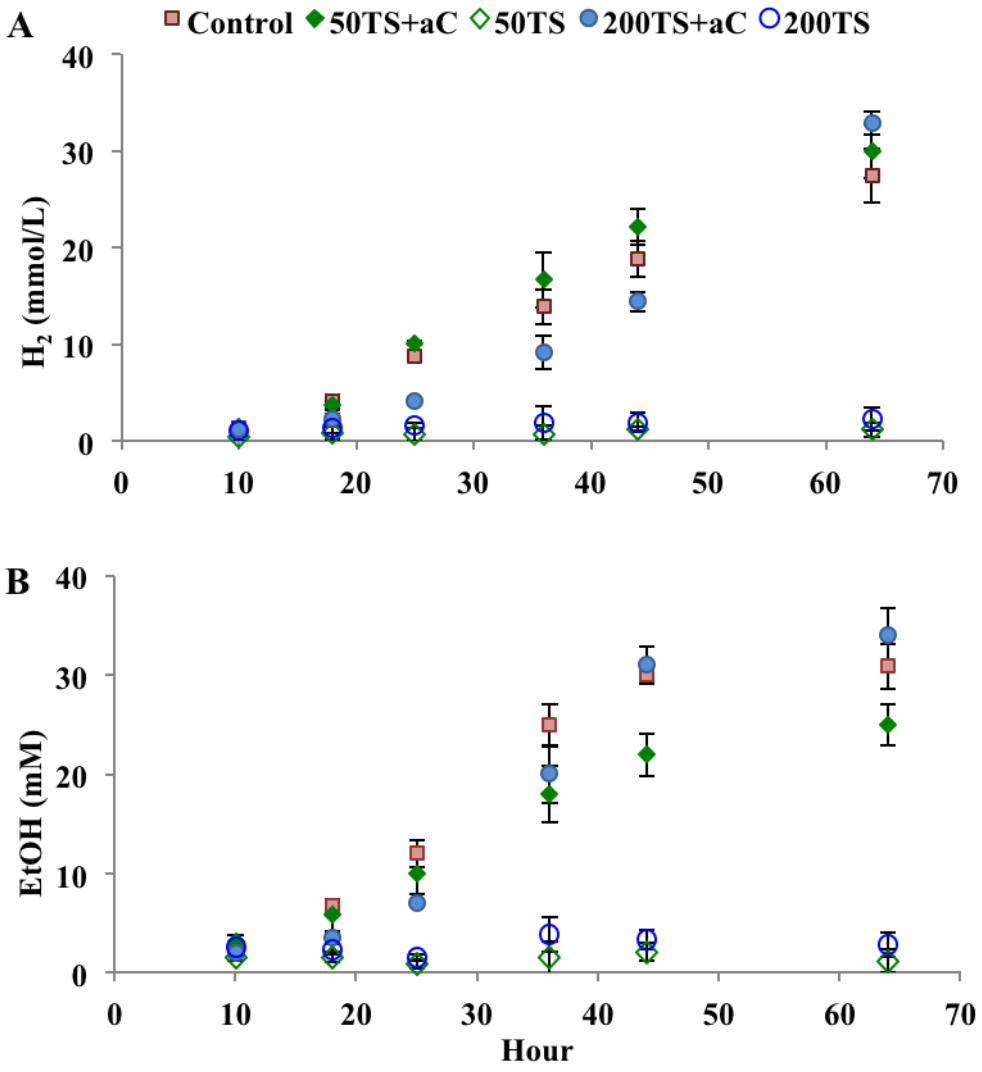

Figure 4. Synthesis of A) H_2 and B) ethanol during batch fermentation by *C. thermocellum* on TS media. Three categories of cultures include standard medium with cellulose (control), TS media with (50TS+aC, 200TS+aC) and without (50TS, 200TS) added cellulose.

3.3.1. Growth of *C. thermocellum* on TS

Growth kinetics of *C. thermocellum* on TS was monitored by optical density measurement when the culture medium was free of insolubles [15]. In anaerobic digestion, volatile suspended solids (VSS) are often used as an indicator of microbial growth [11]. In the present study, it was not possible to quantify cell-growth by optical density or protein concentration due to suspended solids and the high suspended protein (< 30%) content of TS media. Therefore, a qPCR technique was used to quantify

cell growth on the basis of genome copy number. A standard curve (Figure S3, supplementary materials section) based on qPCR amplification of DNA templates (Figure S2, supplementary materials section) extracted from cellobiose grown *C. thermocellum* 1237 cells was developed. The cell-number in a master stock was determined by flow-cytometry, as detailed earlier (Method Section 2.5.2). Growth of *C. thermocellum* on TS media was monitored in terms of log10 of cell-numbers, based on copies of *cpn60* gene amplicons in each sample (Figure 5).

Up to this point TS showed excellent capability to replace the reagent grade mineral salts (magnesium, calcium, iron), vitamins, urea, and yeast extract that are used to prepare a rich growth medium. The total production of H_2 and ethanol by *C. thermocellum* from 50 g/L TS medium was similar to the control medium and higher TS concentrations did not perform better. Therefore, we selected this concentration to examine whether the performance of *C. thermocellum* could be further improved by nutrient supplementation of the TS medium.

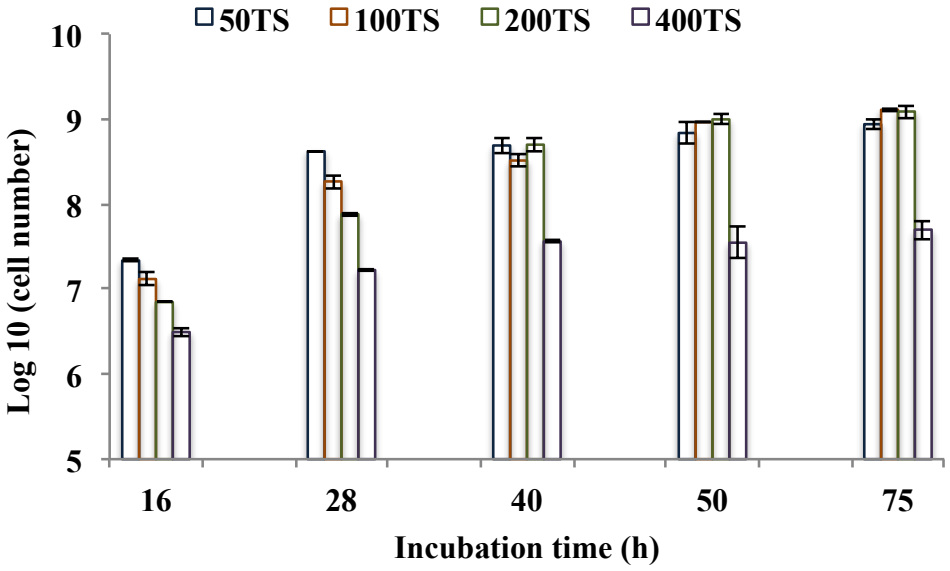

Figure 5. Quantification of cell-growth of *C. thermocellum* 1237 on TS media by qPCR. Time profile of the growth of *C. thermocellum* 1237 in 50, 100, 200 and 400 g/L thin stillage (TS) cultures. The number of cells is based on the copy number of the *cpn60* gene of *C. thermocellum* 1237 estimated by qPCR.

3.4. Magnesium supplementation of TS medium

Compared with the reference medium, TS medium was significantly deficient in some divalent cations (Mg^{++} and Ca^{++}) when added below 200 g/L concentrations. Added magnesium in the optimized medium resulted significantly higher cell-growth and product synthesis by *C. thermocellum* during cellulose fermentation [22]. To check whether an increase in concentrations of divalent cation could improve cell growth and/or end-product concentrations, 0.5 and 1.0 g/L of magnesium chloride (Mg^{++}) were added to 50 g/L TS medium and cultured for 40 hours. Controls consisted of TS medium without added Mg^{++} (Figure 6). Supplementation with 0.5 and 1.0 g/L of Mg^{++} resulted in an average of 35% and 60% increase, respectively, in ethanol concentrations relative to the medium with no added

Mg^{++}. The increase in ethanol concentrations was consistent with the earlier observation of higher amounts of ethanol from cultures with four-fold more TS (Figure 4), which contained correspondingly greater amounts of Mg^{++}. Total H_2 production improved slightly (14% increase) when 0.5 g/L Mg^{++} was added. However, H_2 concentrations dropped to the original level when supplemented with 1.0 g/L Mg^{++} (Figure 6). Levels of lactate accumulation were unchanged, while formate production was always negligible (< 2 mM). This implies the addition of Mg^{++} improved the percentage of conversion of cellulose into ethanol and H_2 without increasing overflow metabolism of *C. thermocellum*.

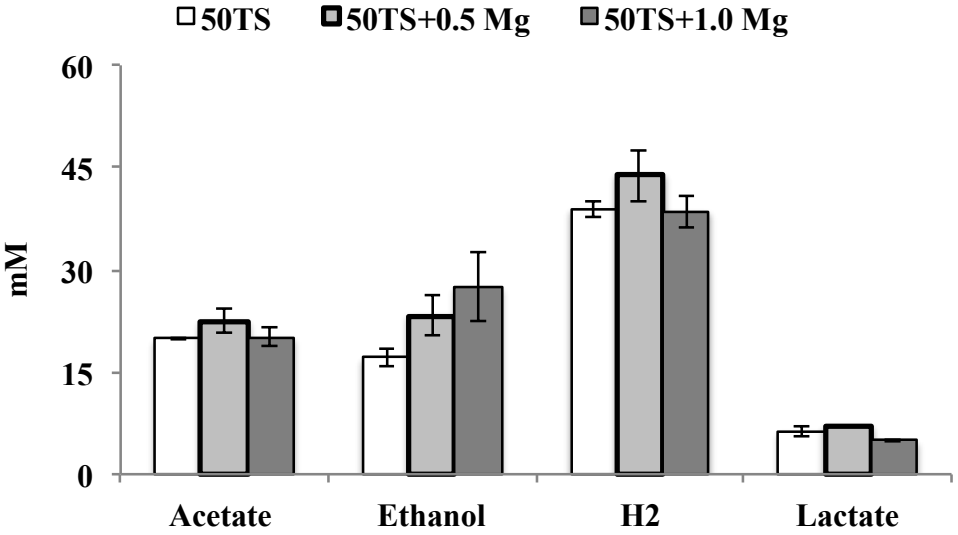

Figure 6. Fermentation end-products synthesized by *C. thermocellum* in 50 g/L TS medium with or without added magnesium (0.5 and 1.0 g/L). Cellulose was added at 10 g/L to all cultures.

4. Discussion

An ethanol distillery co-product, TS was evaluated for its potential as an alternative growth medium for *C. thermocellum*. A wide range of concentration (5 to 400 g/L) was tasted with or without added cellulose as carbon source. Media containing TS at concentrations above 50 g/L displayed lower concentrations of H_2 (interpreted as lower growth, as these are closely correlated), possibly due to adverse effects on cells imposed by a combination of various environmental conditions. As outlined in the Table 1, TS is a complex suspension of grain residues (includes protein and fats), yeast metabolites, and mineral salts. Concentrations of all of these elements increase with greater amounts of TS in the medium, making the fermentation broth more viscous with an oily surface. Possibly the poor liquid-gas mass transfer rate caused over-concentrations of product gases and raised H_2 partial pressures, inhibiting cell growth and/or reducing H_2 production. Due to liquid-to-gas mass transfer limitation in anaerobic processes, H_2 over concentration was 70 times the thermodynamic equilibrium values [23]. H_2 supersaturation, measured with a membrane inlet mass spectrometer during cellobiose fermentation by *C. thermocellum,* increased with growth rate and reached its highest value at maximum growth rate of the culture [24]. Inhibition to growth and H_2 synthesis of

hyperthermophiles by elevated H_2 partial pressures has been well documented [25,26]. In addition, salts may accumulate from pH adjustment of acidic TS media (pH range 5.5–6.0), as higher concentrations of TS are added, which might impart some osmotic stress on cells of *C. thermocellum*.

C. thermocellum is known for its efficient utilization of β-glucans (β-1,4 and β-1,3 glucans) while only a few other substrates such as glucose, fructose and sorbitol supported growth after a prolonged lag phase [27]. Soluble sugars were present in media containing high concentrations of TS, including maltose and glucose [12], which are not metabolizable by *C. thermocellum* under regular conditions. However, growth of *C. thermocellum* on glucose has been demonstrated by some studies [28–30]. It was suggested that medium low in yeast extract limits Glucokinase activities and a positive correlation between the specific activity of Glucokinase, the enzyme that catalyzes glucose metabolism, and concentrations of yeast extract in the growth medium was discovered [28]. Based on these findings, it was expected that glucose would be utilized in TS media where yeast residues are present in abundance. However, our observation and analyses did not detect glucose consumption by *C. thermocellum*.

Active growth phase of *C. thermocellum* was over by 28 h and 40 h in 50 g/L and 200 g/L TS media respectively as depicted by cell-growth data (Figure 6). However, production of both H_2 and ethanol continued after the exponential growth was over (Figure 4). While in the stationary phase of growth, approx. 66% and 72% of H_2 and 74% and 56% of ethanol was evolved by cultures on 50 g/L and 200 g/L respectively. This observation suggests, it would be quite unrealistic from an economic point of view to stop the fermentation with the end of log growth.

The bar-plot in Figure 5 illustrates that, after inoculation, *C. thermocellum* required over 24 h to complete 1 log unit of growth, which is about 30% longer than it takes while growing on the reference medium [17]. This could be due to a lag-period initially experienced by cultures on TS media, which seems to be prolonged in cultures with higher amount of TS. This lag period may have been induced by hydrolysis products in TS, such as glucose and maltose, which are known to inhibit cellulosomes of *C. thermocellum*, although to a lesser extent than cellobiose [31,32]. Also, a non-growth state of *C. thermocellum* was detected upon transferring cultures from cellulose to cellobiose [33]. The same study reported 4% spore formation in the *C. thermocellum* population in absence of vitamins. Vitamins were added to the reference medium by filter sterilization, while TS was autoclaved after preparation. This might have caused severe heat inactivation of vitamin components present in TS, as reported earlier [34].

Several strategies can be approached to further improve the performance of TS as a fermentation medium such as: 1) removal of fats and glycerol from TS to reduce viscosity and improve mass transfer; 2) removal of unusable soluble sugars, which might shorten or eliminate lag time; 3) supplementation with other minerals such as iron or zinc and 4) incorporation of a fraction of TS by filter sterilization to save activities of B-vitamin components. Utilization of glycerol and lactic acid in TS for butanol production [35] could be a viable pretreatment option that could pay for itself. Proposed extraction of other valuables such as corn germ oil and corn fiber oil [36] that do not serve as nutrients for fermentative organisms could improve the performance of TS allowing better liquid-to-gas mass transfer. However, in large-scale fermentation, this grain-oil can be added back in required quantities to serve as a natural antifoaming agent. In our culture bottles containing TS, little to no foaming was observed compared to cultures with the reference medium. In existing ethanol facilities, highly watery TS is recycled to make-up between 20% and 40% of the process water. In this study, the incorporation

of TS to *C. thermocellum* growth medium compensated for up to one-fourth of water volume with 1% cellulose present in the medium. TS, rich in fermentable polysaccharides, could significantly offset the cost of added carbon when used at concentrations over 200 g/L. Thereby, TS could not only potentially replace the cost of growth nutrients, but also could substantially save cost of process water and anti-foaming chemicals in high-scale operations.

5. Conclusions

This study demonstrated the use of TS as an alternative source of low-cost nutrients to support the growth of *C. thermocellum* during cellulose fermentation. Despite lower cell growth rates, ethanol and H_2 concentrations produced by *C. thermocellum* grown in TS media were comparable with those produced by *C. thermocellum* grown in the reference medium. The volumetric H_2 production generated by *C. thermocellum* grown with medium containing a low concentration (50 g/L) of TS matched the volumetric H_2 production by *C. thermocellum* grown in the reference medium, while higher concentrations (200 g/L) of TS resulted in greater synthesis of ethanol. Supplementation of TS-media with Mg^{++} enhanced ethanol production, while H_2 production remained unchanged. TS may be considered a model alternative medium, and represents a readily available industrial waste stream that is rich in microbial growth nutrients. However, the batch-to-batch composition of industrial waste streams vary substantially, depending on the raw materials being used. For consistent performance, the waste stream used for medium formulation should be closely monitored for levels of nutrients, toxic chemicals, and inhibitory substances.

Conflict of interests

The authors declare no conflict of interests.

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