AIMS Energy, 3 (4): 592–611.
DOI: 10.3934/energy.2015.4.592
Received date 29 July 2015,
Accepted date 8 October 2015,
Published date 15 October 2015

http://www.aimspress.com/

Editorial

Investigating the potential of thermophilic species for ethanol production from industrial spent sulfite liquor

Michaela Weissgram [1,2], Thomas Ters [1,3], Hedda K. Weber [1] and Christoph Herwig [2,*]

1 Kompetenzzentrum Holz GmbH, Altenbergerstraße 69, 4040 Linz, Austria
2 Vienna University of Technology, Institute of Chemical Engineering Research Area Biochemical Engineering, Gumpendorferstraße 1A, 1060 Vienna, Austria
3 Vienna University of Technology, Institute of Chemical Engineering Research Area Chemical Engineering, Getreidemarkt 9, 1060 Vienna, Austria

* **Correspondence**: Email: christoph.herwig@tuwien.ac.at; Tel: +43-1-58801-166 400; Fax: +43-1-58801-166 980.

Abstract: Thermophilic microorganisms hold a great potential for bioethanol production on waste biomass, due to their ability to utilize pentoses and hexoses alike. However, to date hardly any data on thermophiles growing directly on industrial substrates like spent sulfite liquor (SSL) are available. This contribution investigates the ability of *Thermoanaerobacter* species to utilize the main sugars in the used SSL (mannose, glucose and xylose) and the effect of process parameters (pH, temperature and sugar concentration) on their growth. Based on these results the strain *T. mathranii* was chosen for further studies. The ability of *T. mathranii* to grow directly on SSL was investigated and the effect of several inhibiting substances on growth was elucidated. Furthermore it was tested whether pretreatment with activated charcoal can increase the fermentability of SSL. The fermentations were evaluated based on yields and specific rates. It could be shown that *T. mathranii* was able to ferment all sugars in the investigated softwood SSL and fermented diluted, untreated SSL (up to 2.7% (w/w) dry matter). Pretreatment with activated charcoal could slightly reduce the amount of phenols in the substrate and thus facilitate growth and ethanol production on higher SSL concentrations (up to 4.7% (w/v) dry matter). Ethanol yields of 0.29–0.44 Cmmol of ethanol per Cmmol sugar were obtained on untreated and pretreated spent sulfite liquor, respectively. These results on an industrial substrate strengthen the claim that thermophilic microorganisms might be the optimal candidates for forest biorefinery.

Keywords: biorefinery; spent sulfite liquor; bioethanol; thermophilic microorganisms; thermophilic

bioprocess; *T. mathranii*

1. Introduction

Worldwide biorefinery concepts focus on sustainable production of liquid transportation fuels and commodity chemicals by conversion of biomass. However, the diversion of farmland or crops for the production of biofuels and bio-based products compromises the food supply causing the dilemma food versus fuel. Several concepts try to circumvent this dilemma by using alternative feedstocks like lignocellulose (2nd generation biofuels) [1,2].

A special case of 2nd generation biofuels uses lignocellulose sugars derived from industrial waste streams, thereby decoupling the biofuel production from agricultural land. Spent sulfite liquor (SSL), an effluent stream from the pulp and paper industry, was used as a substrate for alcohol production using *Saccharomyces cerevisiae* from the early 20th century on [3,4]. Merely a few companies are operating sulfite ethanol mills today due to economical reasons [5]. Although the importance of acid sulfite pulping has decreased in recent years SSL still is one of the most abundant hydrolysates at hand [6]. Hence, it is an ideal cheap raw material for bioprocesses on lignocellulose.

During the acidic sulfite pulping process lignin and part of the hemicelluloses are dissolved in the spent sulfite liquor (SSL), while the cellulose is removed as pulp. The sugars remaining in SSL are released mostly in monomeric form, thus no additional hydrolysis step is needed before fermentation of SSL [2]. Depending on the wood source used for pulping the composition of the SSL can vary significantly. Hardwood SSLs (HSSL) contain more pentoses originating from glucuronoxylans while softwood SSLs (SSSL) show a higher percentage of hexoses mainly from galactoglucomannans. According to Helle [7] xylose can comprise 15% of total sugar in SSSL and more than 50% in HSSL.

Non-genetically modified *S. cerevisiae* is not capable of fermenting C5-sugars into ethanol [8]. Therefore, the bioconversion of HSSL containing high amounts of these sugars was investigated only recently [6,7,9]. Björling and Lindman [10] screened thirty strains of xylose-fermenting yeasts for their ability to produce enhanced ethanol yields in SSL. *Pichia stipitis* CBS 5773, reclassified by Kurtzman and Suzuki [11] as *Scheffersomyces stipitis* was the most promising candidate for industrial applications due to the high ethanol yields. Since then also other organisms like *Candida shehtatae* [12], *Candida guilliermondii* [13], or *Rhizopus oryzae* [14] were evaluated for ethanol production on SSL containing pentoses. *S. cerevisiae*, *Candida tropicalis*, *Pachysolen tannophilus* and *Schizosaccharomyces pombe* were cultivated on HSSL with the addition of xylose isomerase converting xylose to xylulose [15]. Also the use of GMOs like *E. coli* [6] or xylose fermenting *S. cerevisiae* [7,16] and adapted strains of *S. cerevisiae* [17–20] was investigated.

In addition to lignosulfonates and monomeric sugars SSL contains a multitude of low- and high-molecular weight substances inhibiting both biomass production and ethanol fermentation. Among them are degradation products from pentoses and hexoses, namely furfural and hydroxymethylfurfural (HMF), which can affect the specific growth rate [21] and the cell-mass yield on ATP [22] of *S. cerevisiae*. Also organic acids like acetic acid released during hydrolysis of hemicelluloses and formic acid formed during furfural and HMF degradation are common. Raising the pH from 5 to 6 showed no differences on the inhibition of *S. cerevisiae* by SSL suggesting that acetic acid is not the predominant inhibitor for this yeast in SSL [23]. Parajó [24] reported that

phenolic substances originating from lignin degradation, showed a higher inhibiting potential for microorganisms than furfural or HMF. Several different pretreatment strategies have been developed for removal of the inhibiting substances. The most common, economically most feasible pretreatment is overliming with CaO [1,3,15]. Although the exact mechanism of overliming still remains unclear [25] free acid components are removed and furfural is converted to furfuryl acid. Other pretreatment methods include steam stripping of volatile substances like furfural or phenol [12], biodeacidifaction using a *Paecilomyces variotii* strain [9], or the use of ion-exchange resins [26]. Recently Bajwa, et al. [27] described the production of *Pichia stipitis* mutants, mutated by UV mutagenesis, which are more tolerant to inhibitors in HSSL. Using a genetically modified strain of *S. cerevisiae*, Helle and co-workers were able to obtain yields of up to 85% on eucalyptus SSL even without detoxification of the liquor [16].

A rather novel concept for utilization of industrial waste waters are extremophilic bioprocesses. As described before, SSL leaves the process at elevated temperature and low pH value. Thus, thermophilic anaerobic bacteria are a promising option for the production of ethanol from biomass hydrolysates [28] and are especially interesting for integrated processes on SSL. Optimal growth conditions around 60 ℃ reduce the risk of contamination and are much easier to implement into the pulping process due to the reduced energy demand for cooling [29]. Although also thermophilic bacterial strains used for ethanol fermentation can show low tolerances to inhibiting substances there is less information on such inhibitors in literature [30]. Klinke, et al. reported that *T. mathranii* was inhibited by higher concentrations of aromatic substances in alkaline wet oxidized wheat straw [31]. The xylose fermentation of *T. thermosaccharolyticum* was not much influenced by high ethanol or substrate concentrations but severely inhibited by high mineral salt concentrations [32]. On the other hand there is evidence that thermophilic organisms can ferment ethanol on undetoxified pretreated biomass. Mixed cultures with *C. thermocellum* [33] grew on Solka Floc SW40, Larchwood xylan, sulfur dioxide-treated aspen wood, steam-exploded poplar wood and untreated aspen wood chips or other strains [34] grew on unwashed dilute sulfurous acid steam-explosion-pretreated substrates tested (poplar, spruce, miscanthus, wheat straw, whole corn plants, corn cobs, corn stalks, sugarcane bagasse, sweet sorghum, cotton stalks), as well as on untreated dried distillers grains with solubles (DDGS) and waste paper (*Caldicellulosiruptor sp.* str. only DIB 004C on the last substrate). Georgieva, et al. [35] have shown that *T. mathranii* BG1L1 could be used for continuous ethanol fermentation from undetoxified dilute-acid treated corn stover resulting in ethanol yields of around 0.4 g g^{-1} sugar. Furthermore corn stover hydrolysate [35] and wheat straw hydrolysate [36] were tested as substrates for *Thermoanaerobacter ethanolicus.*

1.1. Novelty and goal

The goal of this contribution is to compare the yields of thermophilic species growing on industrial spent sulfite liquor (SSL) to other processes aiming to produce ethanol from spent sulfite liquor. Furthermore the effect of incubating the spent sulfite liquor with activated charcoal for removal of substances known to inhibit microbial growth is investigated to elucidate the potential of thermophilic bioprocesses on industrial waste substrate.

2. Materials and Methods

2.1. Strains and cultivation

Thermoanaerobacter ethanolicus (DSM 2246), *Thermoanaerobacter mathranii* (DSM 11426) and *Thermoanaerobacterium saccharolyticum* (DSM 8691) were purchased from DSMZ (German Collection of Microorganisms and Cell Culture, Braunschweig, Germany). Culture handling as well as preparation of serum flasks and media was performed in an anaerobic glove box (Coy Laboratory Products, Grass Lake, USA). Shake flask cultivations were performed in a working volume of 50 mL [37] in pressure-resistant 100 mL bottles (LaPhaPack GmbH, Langerwehe, Germany) sealed with natural gum stoppers (Laborgerätebau Ochs, Bovenden, Germany) and incubated in a water bath. Cultures were grown as recommended by DSMZ for *Thermoanaerobacter ethanolicus* (65 ℃, pH 6.8–7.5), *Thermoanaerobacter mathranii* (70 ℃, pH 6.8–7.5) and *Thermoanaerobacterium saccharolyticum* (60 ℃, pH 5.2–5.4). Inoculation was carried out with 10% inoculum volume. Cryostocks were kept in 15% glycerol, at −80 °C.

The screening experiments (pH, temperature, sugar concentration, single sugar screening) were performed in 1.5 mL reaction tubes containing 1mL of culture volume, inside the anaerobe glove box. Tubes were inoculated with 10% (v/v) of log phase culture. For OD measurements 100 µL of sample were transferred to flat bottom microtiter plates at the respective sampling points and measured at 600 nm using a microplate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo scientific, Waltham, MA, USA).

2.2. Media

T. ethanolicus was cultivated on medium 61 from German Collection of Microorganisms and Cell Culture (DSMZ), comprised of tryptone 10.00 g L^{-1}, sucrose 10.00 g L^{-1}, yeast extract 2.00 g L^{-1}, $FeSO_4 \times 7\ H_2O$ 0.20 g L^{-1}, Na_2SO_3 0.20 g L^{-1} $Na_2S_2O_3 \times 5\ H_2O$ 0.08 g L^{-1} and resazurin 1.00 mg L^{-1} as a redox indicator.

The strains *T. mathranii* and *T. saccharolyticum* were cultivated on medium 640 from German Collection of Microorganisms and Cell Culture (DSMZ) containing NH_4Cl 0.90 g L^{-1}, NaCl 0.90 g L^{-1}, $MgCl_2 \times 6\ H_2O$ 0.40 g L^{-1}, KH_2PO_4 0.75 g L^{-1}, K_2HPO_4 1.50 g L^{-1}, tryptone/peptone 2.00 g L^{-1}, yeast extract 1.00 g L^{-1}, trace element solution SL-10 1.00 ml L^{-1}, $FeCl_3 \times 6\ H_2O$ 0.0025 g L^{-1}, D-cellobiose 1.00 g L^{-1}, L-cysteine-HCl $\times H_2O$ 0.75 g L^{-1} and resazurin 0.50 mg L^{-1}.

The trace element solution SL-10 was comprised of HCl (25%; 7.7 M) 10.00 mL L^{-1}, $FeCl_2 \times 4\ H_2O$ 1.50 g L^{-1}, $ZnCl_2$ 0.070 g L^{-1}, $MnCl_2 \times 4\ H_2O$ 0.100 g L^{-1}, H_3BO 0.036 g L^{-1}, $CoCl_2 \times 6\ H_2O$ 0.19 g L^{-1}, $CuCl_2 \times 2\ H_2O$ 0.002 g L^{-1}, $NiCl_2 \times 6\ H_2O$ 0.024 g L^{-1} and $Na_2MoO_4 \times 2\ H_2O$ 0.036 g L^{-1}.

2.3. Data evaluation

Significance of univariate results was tested using the Student t-Test. Multivariate data evaluation was focusing on the two-dimensional representations of the dependencies of biomass formation on the process parameters pH and temperature. Multivariate data analysis was carried out using Modde 8 (Umetrics, Malmö, Sweden). For evaluating datasets containing several responses

PLS (partial least squares) was used. The predictive power of a PLS model is given by Q^2 (the cross validated R^2), being a measure of how well the model will predict the responses for new experimental condition [38]. Values close to 1 for both R^2 and Q^2 (at least > 0.7) indicate very good model with excellent predictive power.

2.4. Softwood spent sulfite liquor (SSL)

Softwood (spruce) spent sulfite liquor from two different stages of the evaporation process was used for the experiments. One SSL contained 33% (w/w) dry matter (DM), the other contained 60% (w/w) dry matter. The carbohydrate content ranged from about 19% of the total solids for the 33% batch and about 16% of the 60% batch. The carbohydrate composition of these two batches varied as shown in Figure 2. The 33% (w/w) dry matter batch was used as benchmark; therefore, the calculations considering the inhibitors and the sugar content were based on the specifications of this version. Most of the experiments however, were performed with the 60% (w/w) DM, unless indicated otherwise. The composition of the sugar fraction of SSL is shown in Table 1.

Table 1. Carbohydrate composition in SSL from the different evaporation stages (33% solids and 60% solids, resp.).

	SSL 33%	SSL 60%
Cellobiose	1.0%	0.1%
Glucose	15.0%	18.7%
Xylose	18.3%	17.9%
Galactose	8.3%	6.2%
Rhamnose	0.8%	1.2%
Arabinose	3.4%	3.7%
Mannose	53.1%	52.2%
Total	100.0%	100.0%

2.5. Pretreatment of SSL

SSL was pretreated by incubation with granulated activated charcoal (1–3 mm, from turf, Carl Roth GmbH, Karlsruhe, Germany) at 80 ℃ for 15 minutes [39]. For fermentations on 2.2–2.7% SSL, a 10% dry matter solution was pretreated. For higher concentrations, a 40% dry matter SSL solution was prepared and pretreated. The amount of charcoal was fixed 1:1 to the sugar concentration in the SSL, meaning that to a 10% DM SSL dilution, containing approximately 25 g L^{-1} of sugar, 25 g L^{-1} of were added. After incubation with charcoal the stirring was stopped and most of the activated charcoal settled to the ground immediately. The liquid was decanted carefully into a funnel lined with cellulose filter paper grade 1 (10–11 µm pore size) and the filtrate was collected in a fresh bottle.

Then this pretreated SSL was added to the media as a sugar source, thereby diluting it to the desired final concentration.

The UV absorption of the SSL-samples at 280 nm was determined before and after the pretreatment to investigate the influence of the pretreatment on the content of aromatic lignin derived substances using HPSEC. The content of phenolic OH-groups in the SSL was determined with the Folin-Ciocalteu method [40]. Results were expressed in μmol g^{-1} vanillin. The absorbance was measured at 760 nm.

2.6. Screening for effect of single inhibitors on T. mathranii

To test the effect of inhibiting substances in 33% (w/w) SSL the following concentrations of inhibitors were tested: furfural (0.003 g L^{-1}), HMF (0.039 g L^{-1}), sulfonated lignin (25 g L^{-1}), and a phenol mix (0.127 g L^{-1} and 0.0127 g L^{-1}). The phenol stock solution consisted of vanillin (10 g L^{-1}), vanillic acid (10 g L^{-1}), apocynin (25 g L^{-1}), homovanillic acid (25 g L^{-1}), coniferyl aldehyde (4.9 g L^{-1}), hydroquinone (25 g L^{-1}), catechol (25 g L^{-1}) and 4-hydroxybenzoic acid (2.5 g L^{-1}). The test was performed in Hungate-tubes (Bellco Glass Inc., Vineland, NJ, USA), containing 9 mL of medium supplemented with the respective inhibitors and inoculated with 1 mL of growing culture leading to a final volume of 10 mL.

2.7. Bioreactor set-up and cultivation conditions

Batch experiments were performed in a 2 L table-top bioreactor (Applikon B.V., The Netherlands) at working volumes of 0.8 L and 1 L, respectively.

Pre-cultures of *T. mathranii* were grown at 65.0 ± 0.5 ℃ in shake flasks as previously described.

Sterilization was performed by autoclaving the fully assembled bioreactor containing the medium at 121 ℃ for 20 min. Sterile, anaerobic solutions of cysteine–HCl, trace elements and vitamins were added separately after the sterilization. For reference fermentations the carbon source (a 9 g L^{-1} sugar mix comprised of mannose (5.63 g L^{-1}), glucose (1.53 g L^{-1}) and xylose (1.88 g L^{-1}) resembling the amount of sugar in SSL with about 5% dry matter) was added after sterilization. For cultivations with SSL, however, the carbon source (the respective amount of SSL plus respective amount of glucose, adding up to the final concentration of 9 g L^{-1}) was directly added to the medium for autoclaving, while an anaerobic sterile stock solution containing NH_4Cl, tryptone and yeast extract was added separately after the sterilization.

Prior to inoculation, the bioreactor system, as well as the tubing and the solutions were made anaerobic by flushing them with N_2 for five minutes.

All fermentation parameters and variable pump set-points were controlled by using the process information management system Lucullus 3.1 (SecureCell AG, Schlieren, Switzerland). The cultivation parameters in the bioreactor were 65.0 ± 0.5 ℃ and 150 rpm of agitation. The N_2 inlet flow was adjusted to 0.12 L min^{-1}. The pH was measured by using a pH probe (Mettler-Toledo GmbH, Vienna, Austria) and kept constant at designated values by applying anaerobic 2.0 M NaOH. The pH probe was calibrated at the respective working temperature. Addition of base was performed by using a peristaltic pump (Ismatec SA, Glattburg, Switzerland) and recorded gravimetrically. Oxidation reduction potential (ORP) was measured by using a redox probe (Mettler-Toledo GmbH,

Vienna, Austria). The offgas was cooled by a condenser to reduce stripping of ethanol. H_2 and CO_2, were detected individually via serially applied gas analyzer systems (BlueSens gas sensor GmbH, Herten, Germany). H_2 measurements were corrected in respect to the offgas composition, according to the manufacturer's information. The N_2 inflow rate was controlled by using a mass flow controller (Brooks Instrument, Matfield, USA).

Fermentations were inoculated with 10% (v/v) of culture suspension. The cultures were aseptically and anaerobically transferred into the bioreactor by using a gas-tight syringe.

2.8. Analytical methods

2.8.1. Biomass determination

Cell dry weight (CDW) was determined in quadruplicates by transferring 10 mL of fermentation broth in pre-weight reaction tubes. The reaction tubes were centrifuged at 4000 rpm for 20 min (centrifuge Signum 4K15, rotor 11156). The supernatant was discarded, while the cell pellet was resuspended in 5 mL of distilled water. Then the tubes were centrifuged at 5000 rpm for 10 min. Again the supernatant was discarded and the cell pellet was dried for 72 h at 100 ℃. Cell pellet dry mass was determined gravimetrically.

2.8.2. Elementary composition

The mean elementary composition (carbon (C), hydrogen (H), nitrogen (N), oxygen (O), phosphorus (P) and sulfur (S)) of *T. mathranii* was determined by analyzing steady state conditions (Mikroanalytisches Laboratorium, University of Vienna, Vienna, Austria). The elementary composition was used for the calculation of the mean molar weight of the biomass. The elementary composition of the *T. mathranii* biomass cultivated on sugar mix was $CH_{1.86}O_{0.489}N_{0.238}S_{0.005}P_{0.013}$ with a molar mass of 26.31 g $Cmol^{-1}$.

2.8.3. HPLC analysis

The concentrations of sugars, alcohol and organic acids in the culture supernatant were analyzed by high performance liquid chromatography (HPLC) (Agilent 1100 Series, USA) using a SUPELCOGEL C-610H column (9 μm particle size, 300 × 7.8 mm, Sigma Aldrich, USA) at 30 ℃, with 0.1% (v/v) H_3PO_4 in distilled water (traces of NaN_3) as mobile phase (0.5 mL min^{-1}), followed by a refractive index detection [41].

2.8.4. HPSEC analysis

The UV absorption of the SSL-samples at 280 nm was determined before and after the pretreatment to investigate the influence of the pretreatment on the content of aromatic lignin derived substances. The molar mass distribution of the lignosulfonates was also determined in treated and untreated SSL to see if the pretreatment is selective for a distinct molar mass range. Therefore, the samples were diluted with 10 mM NaOH to a concentration of around 1 mg mL^{-1} DM and analyzed by high performance size exclusion chromatography (HPSEC) (Agilent 1200 Series, USA) using a

TSKgel PWH (7.5 mm × 7.5 cm, 3 μm) guard column followed by a TSKgel G5000PW (7.5 mm × 30 cm, 17 μm), a TSKgel G4000PW (7.5 mm × 30 cm, 17 μm) and a TSKgel G3000PW (7.5 mm × 30 cm, 12 m) at 40 ℃ with 10 mM NaOH as mobile phase (1 mL min^{-1}). Detection was at 280 nm using a diode array detector. The HPSEC system was calibrated with sodium polystyrene sulfonate reference standards (PSS Polymer Standard Services, Germany) with the following molar masses at the peak maximum: 78,400 Da, 33,500 Da, 15,800 Da, 6430 Da, 1670 Da, 891 Da and 208 Da.

3. Results and Discussion

3.1. Screening for optimal pH, temperature and effect of sugar

First the effect of pH, temperature and sugar concentration on the growth of the selected microorganisms was analyzed. For theoretical implementation into the pulping process the aim was to find a microorganism thriving at elevated temperature and low pH. Furthermore the effect of sugar concentration was investigated to show whether high concentrations of sugar mix lead to substrate inhibition. Experiments were performed in the 1 mL scale as described above.

For the investigation of the influence of these factors on the growth of the selected microorganisms a multivariate design of experiments was used. The set of experiments was determined using the software Modde 8 and is shown in Table 2. For the composition of the sugar mix see Table 1.

Table 2. Experimental design used to screen the influence of the factors pH, T and sugar concentration.

DoE condition	low			center			high		
	T (℃)	pH	sugar mix (g L^{-1})	T (℃)	pH	sugar mix (g L^{-1})	T (℃)	pH	sugar mix (g L^{-1})
T. saccharolyticum	50	4	5	60	5.5	18	75	7	36
T. ethanolicus	50	4	5	60	5.5	18	75	7	36
T. mathranii	50	4	5	60	5.5	18	75	7	36

The obtained results were analyzed statistically using the software Modde 8. Influence of the factors pH, temperature and sugar concentration was evaluated and contour-plots for each strain were made. In the statistical analysis of the factors pH, temperature and sugar concentration it could be shown, that pH and temperature had a significant effect. No effect could be verified for high sugar concentrations. Model quality was investigated by looking at R^2, Q^2 and model reproducibility and found to be a valid model (see supplementary data).

The following plots show the effect of pH and temperature on the respective strains with growth ($OD_{600\ nm}$) used as a response (Figure 1).

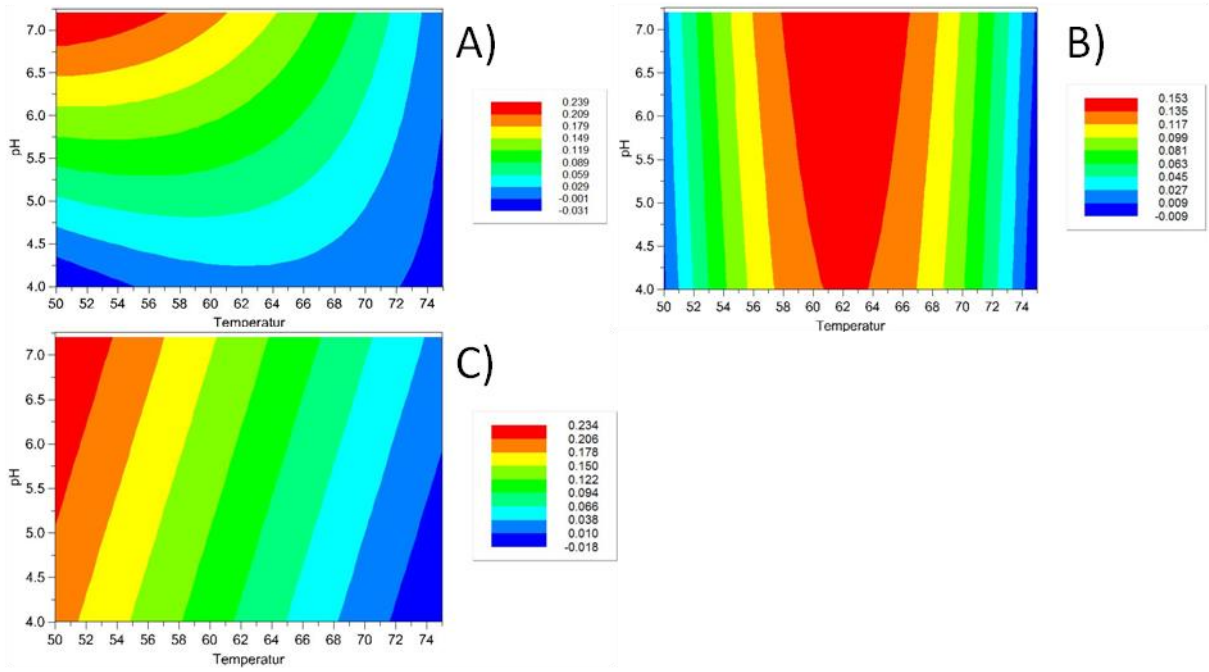


Figure 1. Effect of pH and temperature on cell growth ($OD_{600\ nm}$). A) *T. ethanolicus;* B) *T. mathranii;* C) *T. saccharolyticum;* at 18 g L^{-1} sugar concentration.

As shown pH and temperature affect the growth of all three strains, however the response to the individual factors is different for all strains:

- *T. ethanolicus* is sensitive to decreasing pH levels and rising temperature and has an optimum which lies clearly at pH 7 and between 50 and 58 ℃.
- *T. mathranii* can grow on a wide pH range between pH 4 and pH 7, while it is rather temperature sensitive. The temperature optimum for *T.* mathranii was found to be between 59 and 65 ℃, instead of the previously reported 70 ℃. Hence our later fermentations were performed at 65 ℃.
- *T. saccharolyticum* on the other hand is more sensitive to temperature than to pH, having its optimum cultivation parameters between pH 4 and pH 7 with a temperature range between 50 ℃ and 56 ℃.

3.2. Screening for utilization of single sugars

To investigate the utilization of the various sugars found in SSL, the respective media were supplemented with 5 g L^{-1} of single sugars (arabinose, cellobiose, galactose, glucose, mannose rhamnose, xylose) and the growth on these sugars, as well as the uptake of the respective sugar were investigated using OD and HPLC measurement. Experiments were performed in the 1 mL scale as described above. Harvest time points were 20 h for the *T. ethanolicus* and *T. mathranii* strains and 40 h for *T. saccharolyticum*. The experiments are also summed up in Table 3.

Table 3. Experimental design to screen the for utilization of single sugars arabinose (Ar), cellobiose (Cb), galactose (Ga), glucose (Gl), mannose (Ma), rhamnose (Rh) and xylose (Xy).

Name	Conditions			Sugars	Medium
	T (℃)	pH	C-conc. (g L^{-1})	Ar, Cb, Ga, Gl, Ma, Rh, Xy	
T. saccharolyticum	60	5.4	5	all	640
T. ethanolicus	60	7.2	5	all	61
T. mathranii	60	7.2	5	all	640

As it can be seen in Figure 2 all three strains grew on the three main substrates available in SSL (mannose, glucose and xylose). However, *T. mathranii* showed a much higher growth rate and thus higher OD after 20 h than the other two strains and was thus selected for further investigation by fermentations in a lab scale bioreactor.

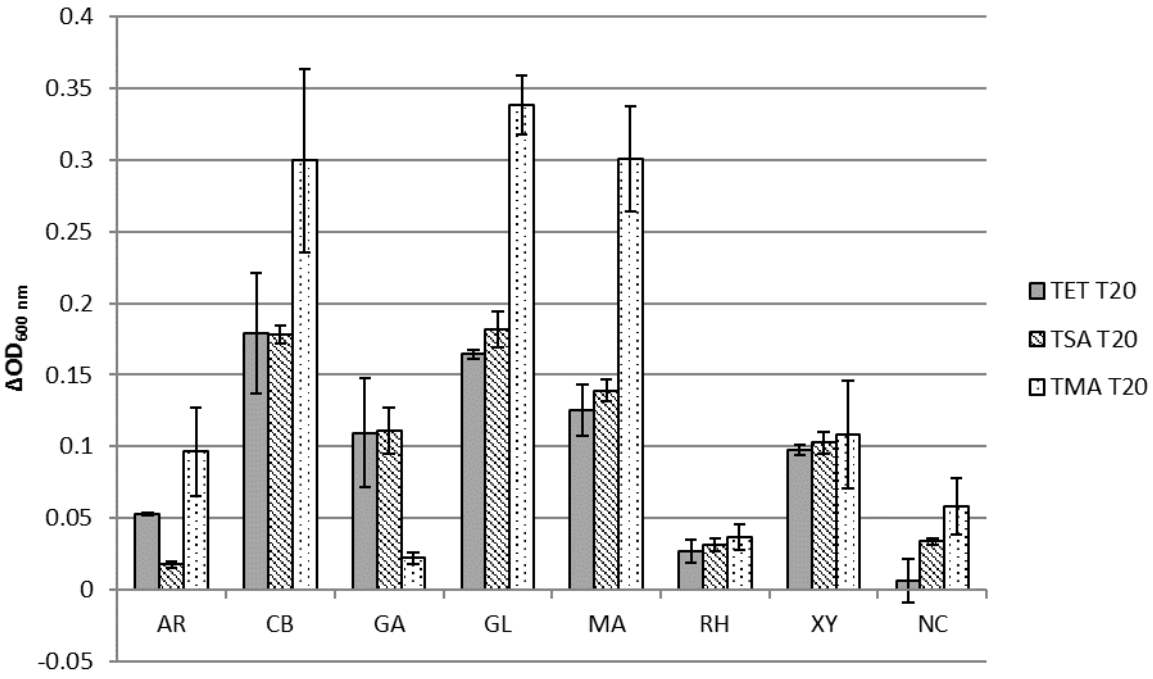


Figure 2. Utilization of various sugars represented by change of optical density $OD_{600\ nm}$: arabinose (AR), cellobiose (CB), galactose (GA), glucose (GL), mannose (MA), rhamnose (RH) and xylose (XY) by the three strains *T. ethanolicus* (TET), *T. saccharolyticum* (TSA) and *T. mathranii* (TMA).

The results were summed up in a table (Table 4), also comparing the results obtained in our lab with results from literature. It could be shown, that most of the sugars are utilized by the thermophilic microorganisms, only rhamnose seems to be no suitable substrate. Our study identified additional sugar utilization as a clear novelty of our work (using t-test, $\alpha = 5\%$).

Table 4. Results from single sugar screening compared with literature [42].

#	Strain	Arabinose	Cellobiose	Galactose	Glucose	Mannose	Rhamnose	Xylose
1	TSA- DSM 8691	n	y	y	y	y	n	y
2	TET- DSM 2246	Y	y	y	y	Y	n	y
3	TMA- DSM 11462	y	Y	n	y	y	n	y

y = grows well, as described in literature;
Y = grows well, novel finding, not mentioned in literature;
n = grows weakly.

3.3. Inhibitor screening T. mathranii

Before starting fermentations several individual substances known to hamper microbial growth and to be present in SSL were tested for their effect on *T. mathranii*. It could be shown that some of the potential inhibitors did not have an effect or even promoted growth, however lignosulfonates and certain phenolic compounds are able to inhibit *T. mathranii* (Figure 3).

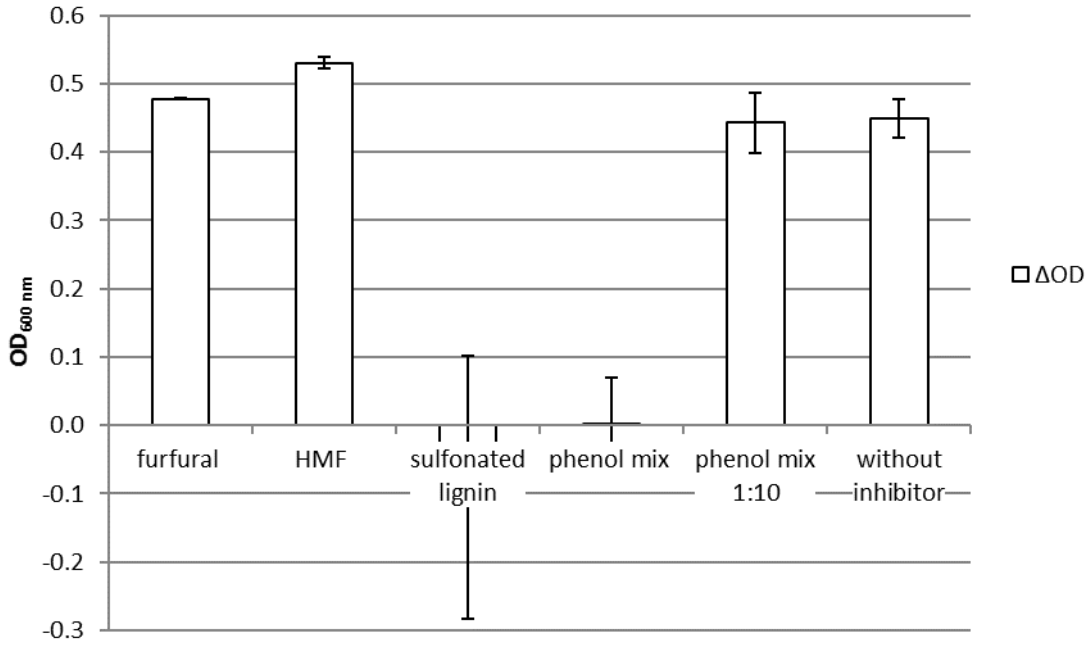


Figure 3. Effect of common inhibitors on *T. mathranii*, showing the difference in absorbance (at 600 nm) directly after inoculation to the absorbance after 20 h of growth for the inhibitors furfural (0.003 g L^{-1}), HMF (0.039 g L^{-1}), sulfonated lignin (25 g L^{-1}), and a phenol mix (0.127 g L^{-1} and 0.0127 g L^{-1}). Inhibitor concentrations were chosen to resemble their estimated concentration in SSL with 33% (w/w) dry matter.

The high standard deviation for sulfonated lignin and the high concentration of phenol mix derives from the freshly inoculated sample. At the inoculation time the substances were not perfectly dissolved, leading to this high deviation, however after 20 h of incubation the deviation was very small again. Still, (almost) no growth occurred in samples provided with 25 g L^{-1} of sulfonated lignin or a high phenol concentration. The inhibitory effect of lignosulfonates on the growth of *T. mathranii* has not been reported previously. According to this result a pretreatment with activated charcoal was chosen over the more frequently used overliming protocol, as activated charcoal is used to bind lignin [43,44].

3.4. Bioreactor cultivations

Several cultivations of *T. mathranii* on reference medium and dilutions of SSL from an industrial process were performed and the ability of *T. mathranii* to generate ethanol from sugars in SSL was investigated. The obtained results were compared to fermentations on SSL and similar hydrolysates found in literature. All yields were calculated in Cmmol per Cmmol, meaning the mmol per C-atom of the respective compound, to assure the comparability of the various carbon sources.

Prior to carrying out fermentations on SSL, a reference-fermentation was performed to ascertain the ability of *T. mathranii* to ferment representative sugars present in the spruce SSL. The C-source added to medium DSMZ 640 for the reference-fermentation comprised of mannose (5.63 g L^{-1}), glucose (1.53 g L^{-1}) and xylose (1.88 g L^{-1}). In this control experiment 3.45 g L^{-1} ethanol was produced. At the end of fermentation 1.93 g L^{-1} organic acid (acetate + lactate) were present in the fermentation broth. A productivity of 0.28 g L^{-1} h^{-1} and a specific rate (q_{EtOH}) of 12.62 Cmmol g^{-1} h^{-1} (Cmmol ethanol per gram biomass per hour) was observed in the reference fermentation. Glucose was the preferred sugar and utilized faster than mannose and xylose (Figure 4A). However, all the sugars were utilized completely.

Serum flask experiments were performed to evaluate at which SSL concentration *T. mathranii* is still able to grow. Concentrations of 2.5%, 3% and 6% (w/v) DM SSL were tested; leading to the result that *T. mathranii* is capable to grow on SSL with a concentration between 2.5 and 3% (w/v) DM SSL.

To confirm this result a fermentation on medium 640 supplemented with 45.5 g L^{-1} of a 60% (w/w DM) SSL solution, leading to a concentration of 2.7% (w/v) DM in the fermentation broth was performed (Figure 4B). In addition to the sugars available in SSL 5 g L^{-1} of glucose were supplemented as C-source. In this experiment 2.12 g L^{-1} ethanol was produced. At the end of fermentation 4.14 g L^{-1} organic acid (acetate + lactate) were present in the fermentation broth. A productivity of 0.1 g L^{-1} h^{-1} and a specific rate (q_{EtOH}) of 5.42 Cmmol g^{-1} h^{-1} were observed. Again glucose was the preferred sugar and utilized faster than mannose and xylose (Figure 4B). All the sugars were completely utilized.

It has been shown in the above section, that lignosulfonates and phenoles have negative effect on *T. mathranii*. Therefore the main interest was the removal of these components, which was performed by treatment with activated charcoal as described in the materials and methods section. For the first fermentation with pretreated SSL a concentration of 2.7% (w/v) dry matter in the fermentation broth was anticipated. Due to the very high viscosity of the 60% (w/w) dry matter SSL not all of it could be recovered from the activated charcoal by filtration, leading to a final concentration of only 2.2% (w/v) dry matter in the fermentation broth. During this fermentation

2.78 g L^{-1} ethanol was produced (Figure 4C). At the end of fermentation 2.71 g L^{-1} organic acid (acetate + lactate) were present in the fermentation broth. A productivity of 0.21 g L^{-1} h^{-1} and a specific rate (q_{EtOH}) of 10.72 Cmmol g^{-1} h^{-1} were observed. Again, all the sugars were utilized completely.

Next it was investigated, whether a higher concentration of pretreated SSL could still be fermented and the next fermentation was run at a concentration of 4.7% (w/v) dry matter in the fermentation broth. During this fermentation 5.37 gL^{-1} ethanol was produced (Figure 4D). At the end of fermentation 2.66 g L^{-1} acid (acetate + lactate) were present in the fermentation broth. A productivity of 0.19 g L^{-1} h^{-1} and a specific rate (q_{EtOH}) of 7.72 Cmmol g^{-1} h^{-1} were observed. This time 16.01 of the provided 18 g L^{-1} of sugars were utilized.

A close comparison of results (Table 5) shows that the produced amount of ethanol in gL^{-1} and the ethanol yield (Cmmol per Cmmol] do not directly show the effect of SSL on the productivity. However, the effect of SSL on the individual cell can be clearly seen when regarding the productivity (g L^{-1} h^{-1}) and the specific rate q_{EtOH}.

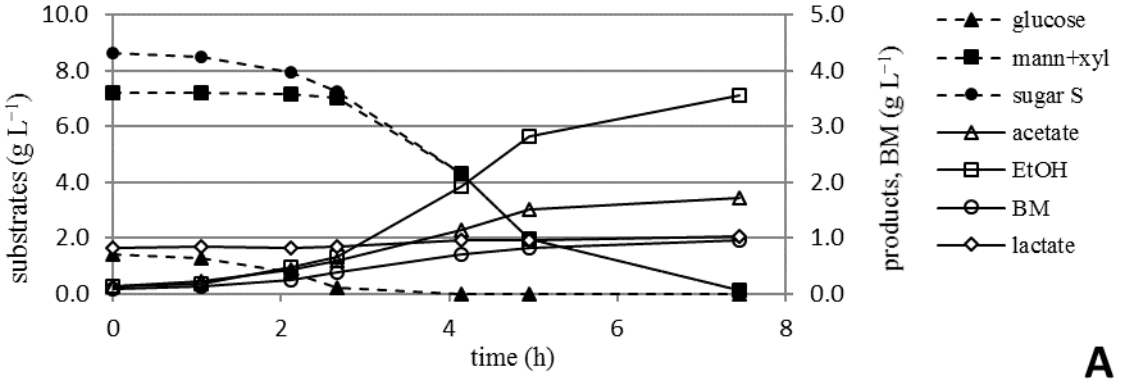


Figure 4A. Reference fermentation on 9 g L^{-1} sugar mix at pH 6.8.

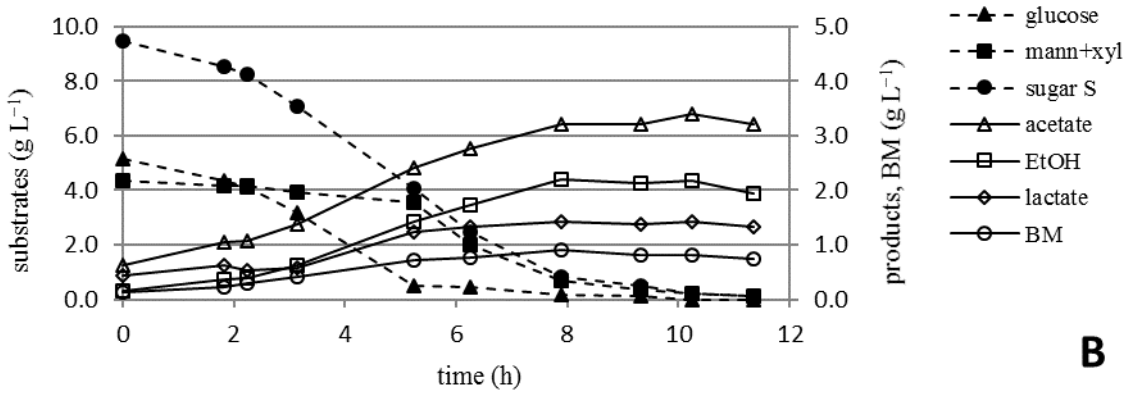


Figure 4B. Fermentation on 2.7% dry matter untreated SSL +5 g L^{-1} glucose.

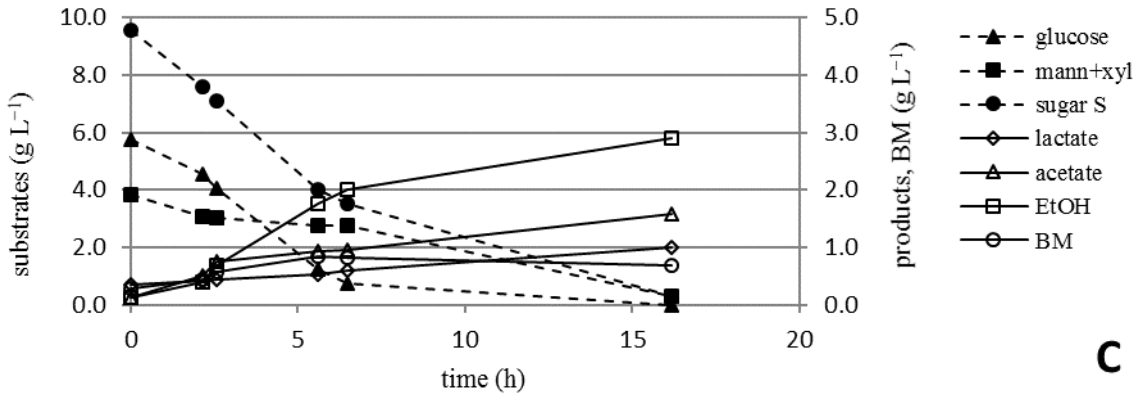


Figure 4C. Fermentation on 2.2% dry matter pretreated SSL +5 g L^{-1} glucose.

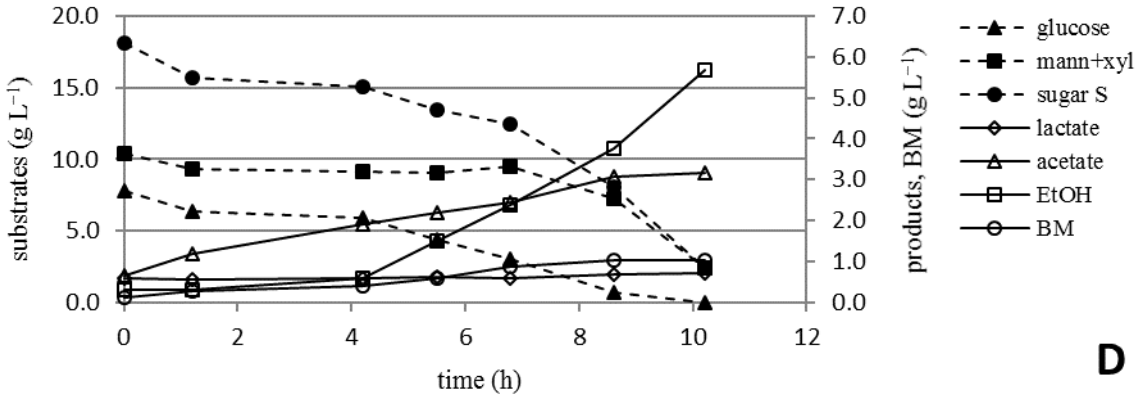


Figure 4D. Fermentation on 4.7% dry matter pretreated SSL +5 g L^{-1} glucose.

Table 5. Summary of fermentation results.

Conditions	util. sugar ($g\ L^{-1}$)	EtOH ($g\ L^{-1}$)	$Y_{EtOH/s}$ ($g\ g^{-1}$)	$Y_{EtOH/s}$ (Cmmol $Cmmol^{-1}$)	$Y_{x/s}$ (Cmmol $Cmmol^{-1}$)	q_{EtOH} (Cmmol $g^{-1}\,h^{-1}$)	prod. (G $L^{-1}\,h^{-1}$)	C-Bal
Reference, pH 6.8	9.0	3.45	0.38	0.50	0.11	12.62	0.28	1.08
2.7% SSL, pH 6.8	9.67	2.12	0.22	0.29	0.09	5.42	0.10	1.10
2.2% SSL pt, pH 6.8	9.25	2.78	0.30	0.39	0.05	10.72	0.21	1.10
4.7% SSL pt, pH 6.2	16.01	5.37	0.34	0.44	0.07	7.72	0.19	0.96
prod.	productivity							
pt	pretreated (activated charcoal)							

When looking at the performance of the individual cell (q_{EtOH}) these results clearly show, that pretreatment can be used to facilitate the utilization of SSL by *T. mathranii*. Although the productivity and specific rate increased after pretreatment, only low concentrations of SSL could be fermented.

3.5. Pretreatment of SSL

Comparison of SSL samples before and after pretreatment showed, that the pretreatment of the SSL led to some small changes in the SSL. The absorption of the samples at 280 nm decreased just around 3%. The molar mass distribution of the SSL is scarcely altered by the pretreatment (see supplementary material). The lignosulfonate in the SSL used had an *Mw (*weight-average molar mass) of 9170 Da, a *Mn* (number-average molar mass) of 1530 Da and polydispersity of 5.99. On the other hand it has been shown that 80 ℃ promote the adsorption of phenols to activated charcoal [39]. Small changes are visible in the region of the low molecular weight substances around a retention time of 25 min. This can be an indication that low molecular weight phenolic substances are removed during the pretreatment. This fact is proven by the determination of the phenolic OH content by the Folin-Ciocalteu method. The pretreatment decreased the content of phenolic-OH groups expressed as vanillin equivalents from 1.74 mmol g^{-1} DM to 1.58 mmol g^{-1} DM. So, a decrease of fermentation inhibitors was observed.

At first it was surprising that only small components, presumably phenoles, were removed during the pretreatment, whereas the large lignin fraction was unaltered, as activated charcoal has previously been used for removal of lignin. However, these results could explain why the inhibitory effect of the full SSL was still so high after pretreatment. Reviewing the pretreatment conditions with respect to lignin, instead of phenoles it was found that according to Mohan [45] activated charcoal has an adsorption capacity of 0.42 mg g^{-1} for lignin. Furthermore, charcoal adsorption of lignin is mainly performed close to room temperature [46–49]. Alternatively application of higher adsorption temperatures between 150 ℃ and 170 ℃ would increase the adsorption significantly [43]. This indicates that by using a temperature optimized for phenol adsorption the lignin adsorption might have been hampered. For an industrial process high temperature adsorption on activated charcoal is an interesting option as SSL leaves the process at 150–170 ℃.

To decide whether further investigation of thermophilic ethanol fermentation utilizing SSL is an interesting option the obtained results (on reference medium and SSL) were compared with values from literature. Several strains have been proposed for the generation of ethanol from lignocellulose biomass with thermophilic microorganisms [50,51]. Comparing the results from fermentations of SSL with the anaerobe strain *T. mathranii* to other studies it can be shown, that the yields are compatible and are comparable with *S. cerevisiae* fermentations (Table 6).

Table 6. Comparing yields of various microorganisms producing ethanol from spent sulfite liquor and similar acid hydrolysates.

Strain	$Y_{p/s}$ ($Cmmol_i\ Cmmol_s^{-1}$)	C-Source	Reference
Caldicellulosiruptor sp.		Lignocellulose	[34]
Clostridium thermosaccharolyticum	0.26	Oak saw dust hydrolysate (1% H_2SO_4)	[52]
Saccharomyces cerevisiae	0.47–0.59	Spent sulfite liquor	[7,16,53]
T. mathranii	0.44	dilutions of SSL	This study

Although *S. cerevisiae* as benchmark organism has a slightly higher yield on SSL than *T. mathranii* in this study, a thermophilic process using *T. mathranii* has several advantages for the

utilization of spent sulfite liquors. Among others *T. mathranii* has a very diverse substrate spectrum, being capable to utilize pentose and hexose sugars, above all mannose and xylose in addition to the more commonly utilized glucose. While in several strains the pentose metabolism is inhibited when glucose is present, simultaneous utilization of glucose and xylose has been observed in several *Thermoanaerobacter(ium)* strains including *T. ethanolicus* [54], *T. thermohydrosulfuricum* [55], *T. saccharolyticum* [56], and *Thermoanaerobacter mathranii* [36]. Other advantages are favorable features of thermophile bioprocesses and the use of a wild-type strain.

4. Conclusions

This study features the concept of forest biorefinery by using spent sulfite liquor as feedstock for the biological production of valuable compounds. Although thermophilic microorganisms as well as their mesophilic counterparts struggle with the inhibiting substances found in SSL thermophilic processes have several advantages over conventional mesophilic fermentations.

Besides the ability of thermophilic microorganisms to use a wide spectrum of substrates, the high process temperature helps avoiding contaminations, saves energy for cooling, and can enable inline product recovery. Our study has shown *T. mathranii* reaches ethanol yields similar to the ones shown by the benchmark organism *S. cerevisiae* on SSL. However, *T. mathranii* seems to be rather sensitive to phenoles and lignosulfonates. Pretreatment using activated charcoal could remove some of the phenoles, but the conditions were not ideal for adsorption of sulfonated lignin. Surprisingly pretreatment could increase the ethanol yields on SSL (0.44 Cmmol $Cmmol^{-1}$) to almost the same level as on a reference medium (0.5 Cmmol $Cmmol^{-1}$). In a nutshell, these results are a robust basis for further projects using SSL as feedstock for biofuels, in particular acetone–butanol–ethanol (ABE) production, which is currently under investigation.

Acknowledgements

The work was performed within the XpremES project, which was funded by the European Regional Development Fund (EFRE) and the province of Upper Austria.

Conflict of interest

The authors declare no conflicts of interest.

References

1. Maniatis K, Chiaramonti D, Thornley P (2012) Framework and perspectives of industrial lignocellulosic ethanol deployment: Introduction to the 1st International Conference on Lignocellulosic Ethanol. *Biomass Bioenerg* 46: 1–4.
2. Fernandes DLA, Pereira SR, Serafim LS, et al. (2012) Second Generation Bioethanol from Lignocellulosics: Processing of Hardwood Sulphite Spent Liquor. In: Pinheiro Lima MA, editor. *Bioethanol*: InTech. pp. 123–152.
3. Nigam JN (2001) Ethanol production from hardwood spent sulfite liquor using an adapted strain of Pichia stipitis. *J Ind Microbiol Biotechnol* 26: 145–150.

4. Frederick WJ, Lien SJ, Courchene CE, et al. (2008) Co-production of ethanol and cellulose fiber from Southern Pine: A technical and economic assessment. *Biomass Bioenerg* 32: 1293–1302
5. Rødsrud G (2011) Biorefining-expanding the sulfite pulping biorefinery concept; Conference presentation at World Biofuels Markets; Rotterdam.
6. Lawford HG, Rousseau JD (1993) Production of ethanol from pulp mill hardwood and softwood spent sulfite liquors by genetically engineered *E. coli*. *Appl Biochem Biotechnol* 39–40: 667–685.
7. Helle SS, Lin T, Duff SJB (2008) Optimization of spent sulfite liquor fermentation. *Enzyme Microb Technol* 42: 259–264.
8. Batt CA, Caryallo S, Easson DD, et al. (1986) Direct evidence for a xylose metabolic pathway in Saccharomyces cerevisiae. *Biotechnol Bioeng* 28: 549–553.
9. Pereira S, Ivanusa S, Evtuguin D, et al. (2012) Biological treatment of eucalypt spent sulphite liquors: a way to boost the production of second generation bioethanol. *Biores Technol* 103: 131–135.
10. Björling T, Lindman B (1989) Evaluation of xylose-fermenting yeasts for ethanol production from spent sulfite liquor. *Enzyme Microb Technol* 11: 240–246.
11. Kurtzman C, Suzuki M (2010) Phylogenetic analysis of ascomycete yeasts that form coenzyme Q-9 and the proposal of the new genera Babjeviella, Meyerozyma, Millerozyma, Priceomyces, and Scheffersomyces. *Mycoscience* 51: 2–14.
12. Yu S, Wayman M, Parekh SK (1987) Fermentation to ethanol of pentose-containing spent sulphite liquor. *Biotechnol Bioeng* 29: 1144–1150.
13. Lai LX (2010) Bioproducts from sulfite pulping: Bioconversion of sugar streams from pulp, sludge, and spent sulfite liquor: University of Washington.
14. Taherzadeh MJ, Fox M, Hjorth H, et al. (2003) Production of mycelium biomass and ethanol from paper pulp sulfite liquor by Rhizopus oryzae. *Bioresour Technol* 88: 167–177.
15. Lindén T, Hahn-Hägerdal B (1989) Fermentation of lignocellulose hydrolysates with yeasts and xylose isomerase. *Enzyme Microb Technol* 11: 583–589.
16. Helle SS, Murray A, Lam J, et al. (2004) Xylose fermentation by genetically modified *Saccharomyces cerevisiae* 259ST in spent sulfite liquor. *Bioresour Technol* 92: 163–171.
17. Guo Z, Olsson L (2014) Characterization and fermentation of side streams from sulfite pulping. *Process Biochem* 49: 1231–1237.
18. Pinel D, D'Aoust F, del Cardayre SB, et al. (2011) Saccharomyces cerevisiae Genome Shuffling through Recursive Population Mating Leads to Improved Tolerance to Spent Sulfite Liquor. *App Env Microbiol* 77: 4736–4743.
19. Pereira S, Sanchez i Nogue V, Frazao C, et al. (2015) Adaptation of Scheffersomyces stipitis to hardwood spent sulfite liquor by evolutionary engineering. *Biotechnol Biofuel* 8: 50.
20. Steensels J, Snoek T, Meersman E, et al. (2014) Improving industrial yeast strains: exploiting natural and artificial diversity. *FEMS Microbiol Rev* 38: 947–995.
21. Boyer LJ, Vega JL, Klasson KT, et al. (1992) The effects of furfural on ethanol production by saccharomyces cereyisiae in batch culture. *Biomass Bioenerg* 3: 41–48.
22. Palmqvist E, Almeida JS, Hahn-Hagerdal B (1999) Influence of furfural on anaerobic glycolytic kinetics of Saccharomyces cerevisiae in batch culture. *Biotechnol Bioeng* 62: 447–454.
23. Olsson L, Hahn-Hägerdal B (1993) Fermentative performance of bacteria and yeasts in lignocellulose hydrolysates. *Process Biochem* 28: 249–257.

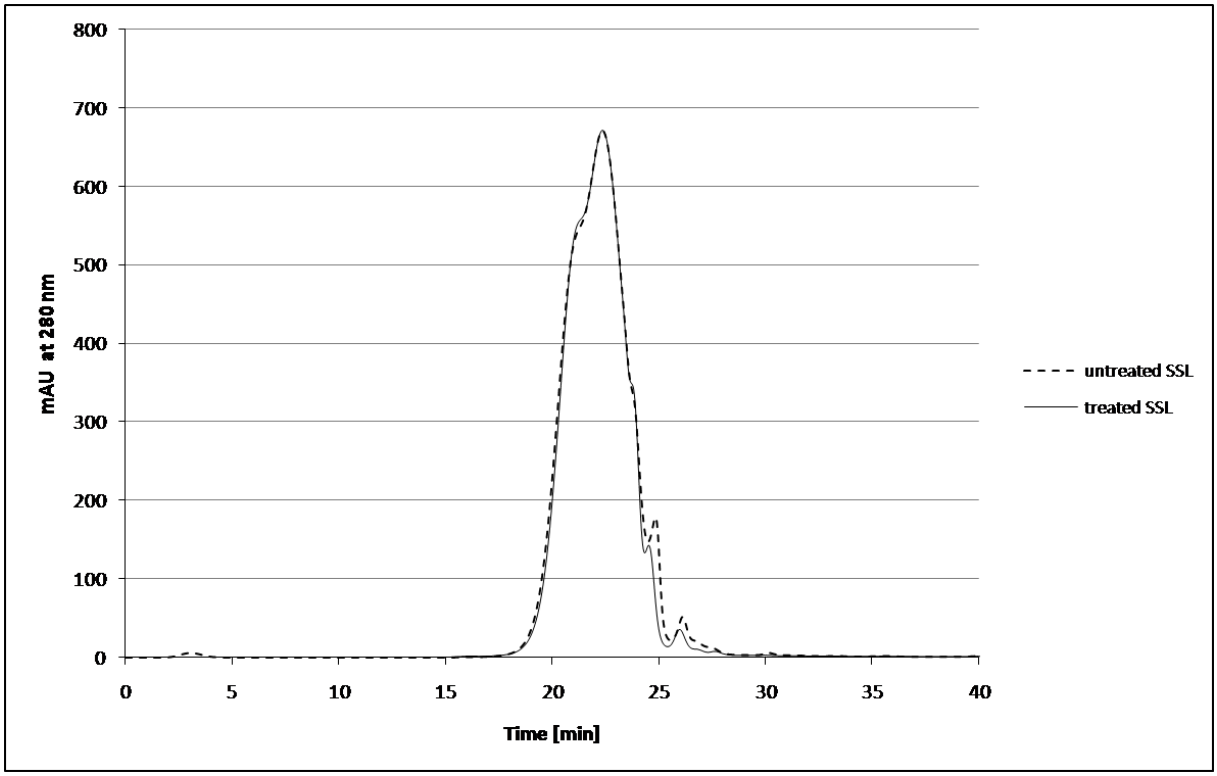
24. Parajó JC, Domínguez H, Domínguez J (1998) Biotechnological production of xylitol. Part 3: Operation in culture media made from lignocellulose hydrolysates. *Bioresour Technol* 66: 25–40.
25. Strickland RC, Beck MJ (1984) Effective pretreatments and neutralization methods for ethanol production from acid - catalyzed hardwood hydrolyzates using Pachysolen tannophilus. Muscle Shoals: T.V.A.
26. Xavier A, Correia M, Pereira S, et al. (2010) Second-generation bioethanol from eucalypt sulphite spent liquor. *Bioresour Technol* 101: 2755–2761.
27. Bajwa PK, Shireen T, D'Aoust F, et al. (2009) Mutants of the pentose-fermenting yeast Pichia stipitis with improved tolerance to inhibitors in hardwood spent sulfite liquor. *Biotechnol Bioeng* 104: 892–900.
28. Lynd LR, Cushman JH, Nichols RJ, et al. (1991) Fuel Ethanol from Cellulosic Biomass. *Science* 251: 1318–1323.
29. Payton MA (1984) Production of ethanol by thermophilic bacteria. *Trends Biotechnol* 2: 153–158.
30. Klinke HB, Thomsen AB, Ahring BK (2004) Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. *Appl Microbiol Biotechnol* 66: 10–26.
31. Klinke H, Thomsen A, Ahring B (2001) Potential inhibitors from wet oxidation of wheat straw and their effect on growth and ethanol production by Thermoanaerobacter mathranii. *Appl Microbiol Biotechnol* 57: 631–638.
32. Lynd LR, Baskaran S, Casten S (2001) Salt Accumulation Resulting from Base Added for pH Control, and Not Ethanol, Limits Growth of Thermoanaerobacterium thermosaccharolyticum HG-8 at Elevated Feed Xylose Concentrations in Continuous Culture. *Biotechnol Prog* 17: 118–125.
33. Ng TK, Ben-Bassat A, Zeikus JG (1981) Ethanol Production by Thermophilic Bacteria: Fermentation of Cellulosic Substrates by Cocultures of Clostridium thermocellum and Clostridium thermohydrosulfuricum. *Appl Environ Microb* 41: 1337–1343.
34. Svetlitchnyi VA, Kensch O, Falkenhan DA, et al. (2013) Single-step ethanol production from lignocellulose using novel extremely thermophilic bacteria. *Biotechnol Biofuels* 6: 31.
35. Georgieva T, Ahring B (2007) Evaluation of continuous ethanol fermentation of dilute-acid corn stover hydrolysate using thermophilic anaerobic bacterium Thermoanaerobacter BG1L1. *Appl Microbiol Biotechnol* 77: 61–68.
36. Georgieva T, Mikkelsen M, Ahring B (2008) Ethanol Production from Wet-Exploded Wheat Straw Hydrolysate by Thermophilic Anaerobic Bacterium Thermoanaerobacter BG1L1 in a Continuous Immobilized Reactor. In: Adney W, McMillan J, Mielenz J et al., editors. *Biotechnology for Fuels and Chemicals*: Humana Press. 99–110.
37. Rittmann S, Herwig C (2012) A comprehensive and quantitative review of dark fermentative biohydrogen production. *Microb Cell Fact* 11: 115.
38. Wold S, Sjöström M, Eriksson L (2001) PLS-regression: a basic tool of chemometrics. *Chemometr Intell Lab* 58: 109–130.
39. Grant TM, King CJ (1990) Mechanism of irreversible adsorption of phenolic compounds by activated carbons. *Ind Eng Chem Res* 29: 264–271.

40. De Sousa F, Reimann A, Björklund M, et al. (2001) Estimating the amount of phenolic hydroxyl groups in lignins. *Proceedings of the 11th International Symposium on Wood Pulp Chemistry* 3: 649–653.
41. Weissgram M, Gstöttner J, Lorantfy B, et al. (2015) Generation of PHB from spent sulfite liquor using halophilic microorganisms. *Microorganisms* 3: 268.
42. Chang T, Yao S (2011) Thermophilic, lignocellulolytic bacteria for ethanol production: current state and perspectives. *Appl Microbiol Biotechnol* 92: 13–27.
43. Gütsch Jenny S, Sixta H (2011) Purification of Eucalyptus globulus water prehydrolyzates using the HiTAC process (high-temperature adsorption on activated charcoal). *Holzforschung* 65: 511–518.
44. Gütsch JS, Sixta H (2012) Regeneration of Spent Activated Charcoals Used for Lignin Removal from Prehydrolysis-Kraft Prehydrolyzates. *Ind Eng Chem Res* 51: 8624–8630.
45. Mohan SV, Karthikeyan J (1997) Removal of lignin and tannin colour from aqueous solution by adsorption onto activated charcoal. *Environ Pollut* 97: 183–187.
46. Mussatto SI, Roberto IC (2004) Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: a review. *Biores Technol* 93: 1–10.
47. Parajó JC, Dominguez H, Domínguez JM (1997) Improved xylitol production with Debaryomyces hansenii Y-7426 from raw or detoxified wood hydrolysates. *Enzyme Microb Technol* 21: 18–24.
48. Maddox IS, Murray A (1983) Production of n-butanol by fermentation of wood hydrolysate. *Biotechnol Lett* 5: 175–178.
49. Montané D, Nabarlatz D, Martorell A, et al. (2006) Removal of lignin and associated impurities from xylo-oligosaccharides by activated carbon adsorption. *Ind Eng Chem Res* 45: 2294–2302.
50. Barnard D, Casanueva A, Tuffin M, et al. (2010) Extremophiles in biofuel synthesis. *Environ Technol* 31: 871–888.
51. Brynjarsdottir H, Wawiernia B, Orlygsson J (2012) Ethanol Production from Sugars and Complex Biomass by Thermoanaerobacter AK5: The Effect of Electron-Scavenging Systems on End-Product Formation. *Energ Fuel* 26: 4568–4574.
52. Liu HS, Hsu HW, Sayler GS (1988) Bioconversion of D-Xylose and Pretreated Oak Sawdust to Ethanol Using Clostridium Thermosacchrolyticum by Batch and Continuous Up-Flow Reactors. *Biotechnol Prog* 4: 40–46.
53. Helle S, Cameron D, Lam J, et al. (2003) Effect of inhibitory compounds found in biomass hydrolysates on growth and xylose fermentation by a genetically engineered strain of *S. cerevisiae*. *Enzyme Microb Technol* 33: 786–792.
54. Carreira LH, Wiegel J, Ljungdahl LG (1983) Production of ethanol from biopolymers by anaerobic, thermophilic, and extreme thermophilic bacteria. I. Regulation of carbohydrate utilization in mutants of Thermoanaerobacter ethanolicus. Medium: X; Size: Pages: 183–191.
55. Cook GM, Janssen PH, Morgan HW (1993) Simultaneous uptake and utilisation of glucose and xylose by Clostridium thermohydrosulfuricum. *FEMS Microbiology Letters* 109: 55–61.
56. Shaw AJ, Podkaminer KK, Desai SG, et al. (2008) Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield. *Proc Natl Acad Sci* 105: 13769–13774.

Supplementary

Supplementary material 1. Model quality parameters.

Strain	Response	R2	R2 Adj.	Q2	N	Reproducibility
TMA	ΔOD e	0.906	0.900	0.886	52	0.949
TET	ΔOD	0.874	0.861	0.813	68	0.985
TSA	ΔOD	0.905	0.901	0.898	62	0.982



Supplementary material 2. HPSEC-chromatograms of untreated and treated SSL.