EVALUATION OF LIGNOCELLULOSIC MATERIAL FOR BUTANOL PRODUCTION USING ENZYMATIC HYDROLYSATE MEDIUM

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Butanol is a promising gasoline additive and platform chemical that can be readily produced via acetone-butanol-ethanol (ABE) fermentation from pretreated lignocellulosic materials. This article examines lignocellulosic material from beech wood for ABE fermentation, using *Clostridium acetobutylicum*. First, the utilization of both C_5- (xylose) and C_6- (glucose) sugars as sole carbon source was investigated in static cultivation, using serum bottles and synthetic medium. The utilization of pentose sugar resulted in a solvent yield of 0.231 $g \cdot g_{sugar}^{-1}$, compared to 0.262 $g \cdot g_{sugar}^{-1}$ using hexose. Then, the Organosolv pretreated crude cellulose fibers (CF) were enzymatically decomposed, and the resulting hydrolysate medium was analyzed for inhibiting compounds (furans, organic acids, phenolics) and treated with ion-exchangers for detoxification. Batch fermentation in a bioreactor using CF hydrolysate medium resulted in a total solvent yield of 0.20 $g_{ABE} \cdot g_{sugar}^{-1}$.

Keywords: lignocellulosic material, ABE fermentation, *Clostridium acetobutylicum*, inhibitors, xylose fermentation, enzymatic hydrolysis

INTRODUCTION

Due to the finite fossil resources, as well as their increasing price levels, there is an ongoing quest for alternative methods for the production of bulk chemicals and fuels. The utilization of renewable raw materials as fermentation substrates is considered as a suitable option. Currently, there is a major focus on the biotechnological production of butanol, which can be used as gasoline additive or gasoline replacement.[1] While doing so, renewable raw materials can serve as fermentation substrates during ABE fermentation, using anaerobic bacterium *Clostridium acetobutylicum*.[2] Usually, the sum of substrate required for the fermentation process accounts for 60% of the overall production cost.[3] Therefore particularly lignocellulosic material, such as agricultural or forestry residue, is a potential feedstock for butanol production, due to its abundance and low cost.[4] Lignocellulosic biomass is composed of cellulose, hemicelluloses and lignin.[5] Pretreatment and enzymatic hydrolysis of biomass allows the release of C_5- and C_6-sugars, while solventogenic clostridia are able to metabolize a great variety of substrates, such as pentoses and hexoses.[6] Nevertheless, the pretreatment of lignocellulose may result in toxic degradation products from the carbohydrate and lignin fraction, which can hamper the fermentation processes. Particularly acetic acid, furfural and 5-hydroxymethylfurfural (HMF), ρ-coumaric acid, vanillin and phenols have been identified as major sources.[7,8]

In this work, the cultivation of *C. acetobutylicum* using single hexose and pentose as carbon source in synthetic P2 medium, as well as mixtures of both sugars will be evaluated. Afterwards, the performance of the production strain DSM 792 in enzymatically derived hydrolysate medium from CF will be analyzed. Additionally, CF medium will be analyzed for potential inhibitory compounds and detoxified using ionic resins. This detoxified (detox.) hydrolysate medium serves as cultivation medium. This study gives information about future possibilities of ABE formation in enzymatically derived cellulose fiber hydrolysate medium from lignocellulose biomass.

EXPERIMENTAL

Methods and chemicals

If not stated otherwise, all chemicals and reagents used in this study were of high purity (p.a.), and purchased from Sigma-Aldrich (Steinheim, Germany). Detailed description of the methods applied in this

study can be found in our previous publication.[9]

Preparation of hydrolysate medium

Beech wood was pretreated by the Organosolv process, after that, crude cellulose fibers were enzymatically decomposed. The conditions for the pretreatment were as follows: 100 min at 170 °C with a solid to liquid ratio of 1:3 (EtOH/H_2O=1:1; with 0.5% H_2SO_4 as catalyst). The resulting hydrolysate medium was analyzed for sugar concentrations and inhibitory compounds. The detoxification of 1 L hydrolysate medium was achieved by using 300 g ion-exchange resins. For the fermentation approach, the hydrolysate medium was filtered through a 0.2 µm vacuum filtration system (VWR, Darmstadt, Germany).

Cultivation of *C. acetobutylicum*

ABE-Fermentation was conducted with *Clostridium acetobutylicum* DSM 792. The culture was acquired from the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany. PYX medium (medium 104b, DSMZ, http://www.dsmz.de) was used as pre-culture medium. 1 mL stock solutions of the strain were kept in 20% glycerol at -80 °C. As inoculum, 10% of a 24-36 h grown culture at 37 °C was used. Fermentations in 100 mL serum bottles were performed statically, using P2 medium with either glucose or xylose as carbon source at a concentration of 60 $g \cdot L^{-1}$. Sugar solutions were prepared separately and added, after autoclaving. Experiments with glucose (60 $g \cdot L^{-1}$) and xylose mixtures (20 $g \cdot L^{-1}$) were performed in a Biostat Q plus bioreactor from Sartorius (Göttingen, Germany) using 400 mL culture media. P2 medium consisted of the following: 1 $g \cdot L^{-1}$ yeast extract, 2.2 $g \cdot L^{-1}$ ammonium acetate, 1 mL antifoam agent, 1 $mL \cdot L^{-1}$ vitamin solution (I) and 5 $mL \cdot L^{-1}$ mineral solution (II). The composition of the solution was as follows: (I) 1 $g \cdot L^{-1}$ p-aminobenzoic acid, 1 $g \cdot L^{-1}$ L-thiamin and 0.01 $g \cdot L^{-1}$ biotin; (II) 40 $g \cdot L^{-1}$ $MgSO_4 \cdot 7\ H_2O$, 2 $g \cdot L^{-1}$ $MnSO_4 \cdot H_2O$ and 2 $g \cdot L^{-1}$ NaCl.

Analytical procedures

Sugar concentrations in the supernatant were measured by high performance liquid chromatography (HPLC), using a refractometric detector. Separation was carried out on a 300 x 8 mm ReproGel Ca^{2+}-column (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) at 80 °C. Ultrapure water with a flow rate of 0.5 $mL \cdot L^{-1}$ served as mobile phase. The injection volume was 20 µL. Phenolic compounds and furans were determined using a 250 mm x 3 mm Eurosher II 100-5 C 18 column (Knauer, Germany) heated at 30 °C. The flow rate was 0.5 $mL \cdot L^{-1}$ using a gradient of methanol and 0.1% formic acid. Organic acids and ABE solvents were determined by gas chromatography (Clarus 560, PerkinElmer, Waltham, USA) using a flame ionization detector and a 30 m Elite-Q PLOT column (PerkinElmer, Waltham, USA). The flow rate for the helium carrier gas was 2 $mL \cdot min^{-1}$ at 180 °C.

RESULTS AND DISCUSSION

Analysis of hydrolysate medium

The evaluation was performed in order to determine to which extend crude cellulose fiber hydrolysate is suitable for ABE fermentation process. The effects of the pretreatment on the lignocellulosic hydrolysate medium were recorded analytically. In Table 1, the results of the determination of cellulose and hemicelluloses amounts of the tested raw materials are given.

The composition of the beech wood chips before pretreatment of the biomass was 46% cellulose and 16% hemicelluloses. After the Organosolv pretreatment, CF was 76% cellulose and 8% hemicelluloses, respectively. The pretreatment allowed the effective removal of recalcitrance of the lignocellulosic biomass and facilitated enzymatic hydrolysis.[10] Other pretreatment methods, such as acid or alkali treatment, might additionally relieve the production of inhibitory compounds, due to the high temperature and long residence time, which are unfavorable in bacterial cultivation.[11]

Known inhibitors in a lignocellulosic hydrolysate medium are thermally derived sugar-degradation products, such as HMF, furfural, acetic, formic and levulinic acid, as well as lignin degradation products.[12] In Table 2, the results of the analysis of the hydrolysate medium are given with the values obtained after the application of ionic resins.

Table 1
Cellulose and hemicelluloses amounts of beech wood and cellulose fibers in % dry weight (DW)

	Cellulose (% DW)	Hemicelluloses (% DW)
Beech wood chips	46	16
Crude cellulose fibers	76	8.0

Table 2
Organic acids, phenolic compounds and furans in g·L^{-1} found in different hydrolysate media and the decrease in %, after detoxification with ion exchangers

Compound	C (g·L^{-1})	Decrease (%)
Acetic acid	13.14	56
Ferulic acid	0	-
Formic acid	0.1923	59
Furfural	0.0037	62
HMF	0.0075	56
Levulinic acid	0	-
ρ-coumaric acid	0.00227	94
Syringaldehyde	0.0828	66
Vanillin	0.0129	67

The amounts of potentially inhibitory compounds found prior to detoxification were relatively low, except for acetic acid with 13.14 g·L^{-1}. In the literature, amounts of 6.53-10.10 g·L^{-1} for hydrolysate media from wheat and barley straw, corn stover and switchgrass were reported.[13] In this process, it was shown that some of the acetic acid present in the medium could be utilized by solventogenic bacteria, as clostridia, during ABE fermentation.[14] However, in other studies, the appearance of acetic acid (3.5 g·L^{-1}) with oxalic acid (6.58 g·L^{-1}) had a negative effect on total xylose consumption.[15] After detoxification, only 44% of the acid was still present. The amounts of HMF and furfural were between 7.5.-3.7 mg·L^{-1} and could be reduced to 56 and 62%, respectively. Hence, furans showed a stimulatory effect on solventogenic clostridia at concentrations of 0.5-2 g·L^{-1},[16] while for *C. acetobutylicum* ATCC 824 furans exerted temporary inhibition at concentrations of 1-2 g·L^{-1}.[17] Interestingly, no ferulic or levulinic acids were found in the CF hydrolysate medium. Previously, Ezeji and co-workers found that the introduction of ferulic acid and *p*-coumaric acid at concentrations of 0.3 g·L^{-1} inhibited growth and ABE production of *C. beijerinckii* BA101.[18] Nevertheless, with detoxification of hydrolysate medium 94% of p-coumaric acid could be removed. The measured formic acid amount was 0.1923 g·L^{-1} and could be decreased by 59%. The concentration of syringaldehyde in the medium was 0.0828 g·L^{-1}. Richmond *et al.* found that syringaldehyde had a negative effect on *C. beijerinckii* 8052, and specifically hampered the ability to metabolize butyric and acetic acids, which are precursors in solvent formation.[19] The concentration of vanillin could be reduced by detoxification to 67%. Although vanillin has an inhibitory effect on multiple clostridia strains, the amounts found in the CF medium are much lower, as reported previously.[20]

Batch fermentation with *C. acetobutylicum*

The production and concentration distribution of a typical ABE fermentation over time, using *C. acetobutylicum* in static cultivation with either glucose (Glu) or xylose (Xyl) as sole carbon source, are shown in Figure 1. The initial growth phase utilization of hexose as carbon source is faster compared to the usage of pentose sugar (Figure 1A) resulting in the maximal optical density (OD) of the clostridial biomass of 5.5 within 30 hours. Growth with xylose utilization requires twice the amount of time until a maximum OD of 3.5 is reached. The lag phase of the microorganisms is overcome after 52 hours of fermentation, whereas exponential growth is initiated. Although growth in both carbon sources is possible, it occurs at a much slower rate with pentose sugars.[21] This behavior is also seen during solvent formation, while the final butanol amount is lower using xylose as carbon source.[22]

Overall, the first butanol formation was detected after 30 h, using glucose as carbon source (Figure 1B). Maximum solvent concentrations were reached after 54 hours, with 2.73 g·L^{-1} butanol, 0.78 g·L^{-1} acetone and 0.38 g·L^{-1} ethanol. Using xylose as carbon source, maximum ABE concentration was as follows: 0.99 g·L^{-1}, 2.59 g·L^{-1} and 0.54 g·L^{-1}.

The results indicated that both primary sugars arising from lignocellulose can be used for solvent formation. However, the conversion rate is lower using pentose sugar. Nevertheless, cultivation in serum bottles depicted the trend of fermentation course, although fermentation was

not fully adequate, due to insufficient allocation of static culture and no pH control.

The results of the growth experiments were the basis for fermentation studies in a bioreactor using media in which both carbon sources were present in the same relation (Figure 2). In conformity with the previous results, fermentation studies were performed with control P2 medium (A-B). Additionally, actual CF hydrolysate medium derived from enzymatic hydrolysis of 100 $g \cdot L^{-1}_{DW}$ beech wood cellulose fibers, with naturally containing sugar fractions (60 $g \cdot L^{-1}$ glucose, 20 $g \cdot L^{-1}$ xylose) was used (C-D).

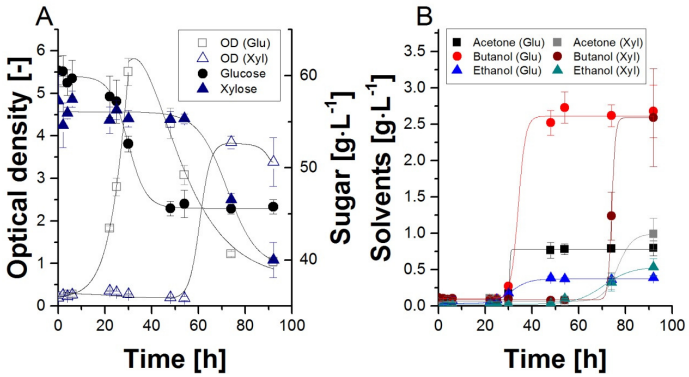

Figure 1: Growth and solvent production of *C. acetobutylicum* in P2 medium with glucose or xylose as carbon source; A. Optical density over time; B. Solvent formation ($g \cdot L^{-1}$) of acetone butanol and ethanol. Static cultivation with 60 $g \cdot L^{-1}$ sugar in 100 mL

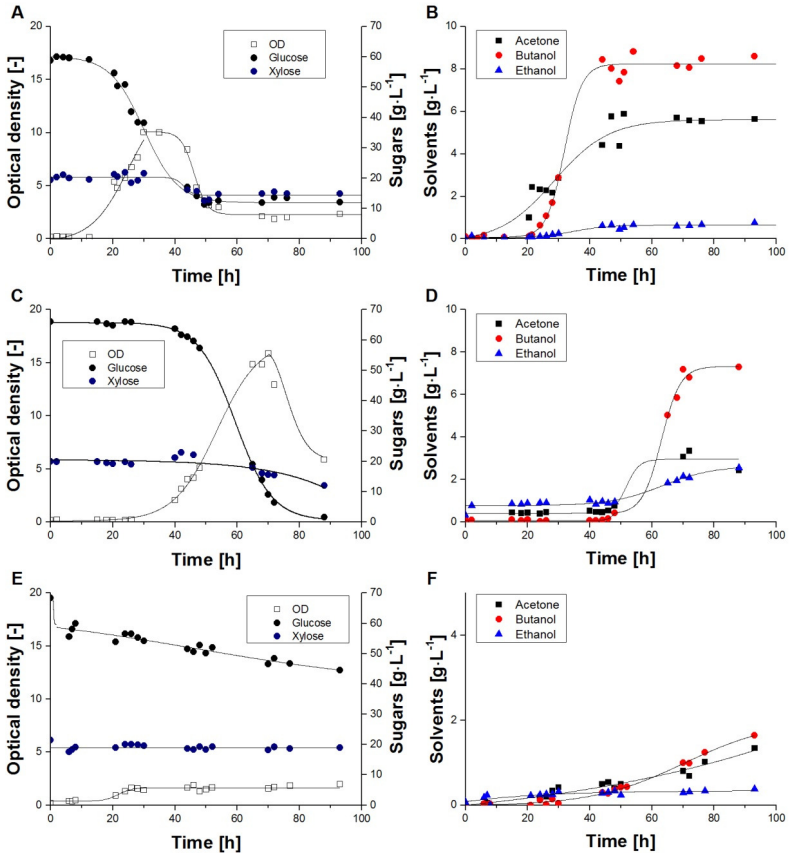

Figure 2: Growth (left) and ABE solvent formation (right) of *C. acetobutylicum* cultivated in different media; (A-B) P2 medium, (C-D) CF hydrolysate, (E-F) detox. CF hydrolysate. Start sugar concentration always 60 $g \cdot L^{-1}$ glucose, 20 $g \cdot L^{-1}$ xylose at 37 °C

Hydrolysate medium derived from pretreated lignocellulosic biomass potentially contains a variety of inhibitors, which might hamper process efficiency during butanol fermentation. Hence, the actual performance of *C. acetobutylicum* DSM 792 was also compared with previously detox. CF hydrolysate medium (E-F).

Synthetic medium containing the same sugar concentration as the hydrolysate was used as a control, without having any additional inhibitors from the pretreatment of raw biomass (Figure 2A-B). Here, *C. acetobutylicum* (DSM 792) could consume all of the initial glucose in the medium within 70 h. In contrast, xylose was first consumed after the glucose concentration reached a trigger concentration of 18 $g \cdot L^{-1}$, which is caused by catabolite repression.[23,24] However, in comparison with the fermentation with hydrolysate medium, an optical density of only 11 was reached. This effect is caused by a failure in pH regulation, inducing an unfavorable environment for bacterial growth. Nevertheless, in this experiment a maximum butanol concentration of 8.45 $g \cdot L^{-1}$ was reached within 45 hours and may serve as sufficient reference for the technical grade hydrolysate. The concentrations of acetone and ethanol were 4.41 $g \cdot L^{-1}$ and 0.62 $g \cdot L^{-1}$, respectively. This corresponds to a yield of 0.18 $g \cdot g_{sugar}^{-1}$ butanol and 0.28 $g \cdot g_{sugar}^{-1}$ solvents. In the hydrolysate medium (Figure 2C-D), *C. acetobutylicum* shows a similar fermentation profile as with standardized media, hence with a slower exponential growth phase compared to P2 medium. In enzymatic hydrolysate medium, the strain consumed all of the available glucose in 88 hours. In the course of xylose consumption, a decline of the pentose concentration is visible, after reaching a glucose concentration below 18 $g \cdot L^{-1}$. In this experiment, a slightly lower amount of butanol was produced compared to P2 medium (7.18 $g \cdot L^{-1}$), which corresponds to a butanol yield of 0.12 $g \cdot g_{sugar}^{-1}$ at a maximum optical density of 15. The amounts of the other solvents in the broth were 3.07 $g \cdot L^{-1}$ acetone and 2.12 $g \cdot L^{-1}$ ethanol, respectively. Interestingly, the fermentation with detox. hydrolysate medium (Figure 2E-F) showed the lowest growth and sugar consumption. None of the xylose and only 25.4 $g \cdot L^{-1}$ of the hexose sugar were consumed. Growth in the medium was possible, but maximum optical density reached during fermentation time was OD 2.0. Due to decreased microbial growth maximum butanol concentration reached in the broth was only 1.64 $g \cdot L^{-1}$, followed by acetone with 1.3 $g \cdot L^{-1}$ and ethanol with 0.4 $g \cdot L^{-1}$. Since all potential inhibitory compounds were removed previously, it is assumed that the decrease in ABE formation is due to the nonselective removal of minerals and vitamins present in the medium during treatment with ion-exchange resins.

CONCLUSION

In preliminary tests, the static cultivation of the production strain DSM 792 with either glucose or xylose as carbon source revealed that growth and solvent formation are possible with both monosaccharides. Hence, when using xylose as sole carbon source, both growth and ABE formation is decelerated.

ABE fermentation was further conducted with media containing both pentose and hexose sugars. The control batch fermentation with standardized medium composition resulted in the production of 13.5 $g \cdot L^{-1}$ of ABE using *Clostridium acetobutylicum* DSM 792. Time-delayed consumption of xylose was possible, after 70% of glucose sugar was consumed. This experiment leads to a maximum ABE yield of 0.28 g g^{-1}.

In this work, it was further shown with comparative fermentation experiments that both growth and solvent production are possible using crude beech wood cellulose hydrolysate medium without detoxification of the hydrolysate medium. In untreated CF hydrolysate medium, the maximum butanol concentration reached was 7.2 $g \cdot L^{-1}$ at a solvent yield of 0.20 $g \cdot g_{sugar}^{-1}$. Consequently, the great potential of CF was shown having both C5- and C6-sugars, which might increase process efficiency using a low cost lignocellulosic material. Interestingly, fermentations with detox. medium resulted in a reduced ABE production, of only 3.4 $g \cdot L^{-1}$. It is assumed that growth-relevant trace minerals are removed during detoxification. Thus, Organosolv and enzymatic pretreatments of beech wood are sufficient for subsequent production of microbial ABE solutes.

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