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Butanol Production from Lignocellulosic-based Hexoses and Pentoses by Fermentation of *Clostridium Acetobutylicum*

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The Acetone-Butanol-Ethanol (ABE) fermentation is receiving renewed interest as a way to upgrade renewable resources into valuable base chemicals and liquid fuels. Abundance and un-competitiveness with food sources are desired features of a potential substrate, and they are met by lignocellulosic biomass.
This contribution is about the characterization of the ABE fermentation by *C. acetobutylicum* DSM 792 using sugars representative for hydrolysis products of lignocellulosic biomass: hexoses (glucose and mannose) and pentoses (arabinose and xylose).
Batch fermentations of single sugars were carried out. The conversion process was characterized as a function of time in terms of biomass, acids and solvents concentrations as well as pH and total organic compounds. *C. acetobutylicum* was able to convert all investigated sugars. The conversion into solvents was strongly enhanced by the presence of $CaCO_3$ in the fermentation medium.

1. Introduction

Interest in biofuel production has recently grown due to continuous depletion of worldwide oil deposits, awareness in greenhouse gas emissions during combustion of fossil fuels and potential impacts of this activity on global warming. Butanol is a good candidate as a biofuel for its interesting features (Dürre, 2008; Lee et al., 2008): lower vapour pressure, blending with either gasoline or diesel at any fraction, energy content close to that of the gasoline, fuelled to current configuration of engines without any retrofitting.
Butanol - currently manufactured with petroleum feedstock (Oxo process) - may be produced from renewable resources (biomass) by the acetone butanol ethanol (ABE) fermentation. Among the clostridia - saccharolytic butyric acid-producing bacteria - there are a number of species capable of producing significant amounts of neutral solvents during the later stage of batch fermentations under appropriate operating conditions (*Clostridium saccharoperbutylacetonicum*, *C. acetobutylicum*, *C. beijerinckii*, *C. aurantibutyricum*). Solventogenic clostridia are anaerobic strains able to metabolize a great variety of substrates, pentoses, hexoses, mono-, di- and polysaccharides (Flickinger and Drew, 1999). Typically, the strains selected in industrial fermentation depend on the nature of the raw material available on the market, the required ratio between the solvents, the need for additional nutrients and

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phage resistance (Ross, 1961). *C. acetobutylicum* has been successfully adopted for the production of acetone and butanol. Under batch conditions the fermentation process of solvent-producing clostridium strains proceeds with the production of cells, hydrogen, carbon dioxide, acetic acid and butyric acid during the initial growth phase (acidogenesis). As the acid concentrations increase (pH decrease), the metabolism of cells shifts to solvent production (solventogenesis) and acidogenic cells – able to reproduce themselves - shift to the solventogenesis state with a morphological change. During solventogenesis the active cells become endospores unable to reproduce themselves. Two different physiological states must be taken into account for Clostridia: one for the acidonegenic phase, and one for the solventogenic phase.

The ABE fermentation is receiving renewed interest as a way to upgrade renewable resources into valuable base chemicals and liquid fuels (Cascone, 2008). Major challenges with ABE fermentation concern low solvent concentrations, yields and productivities due to butanol toxicity to microbial cells (Ezeji et al., 2003). Recent developments of molecular techniques applied to solventogenic microorganisms in combination with advances in fermentation technology and downstream processing have contributed to improve feasibility and competitiveness of the ABE fermentation process. The ABE concentration and productivity in a typical batch fermentation by solventogenic *Clostridium* species can be increased to values between 13 to 18 g/L and 0.2 to 0.3 g/L/h, respectively (Ezeji and Blaschek, 2008). Modest increases in ABE titer and productivity can be achieved by supplementing acetate (Chen and Blaschek, 1999) or butanol (Junne et al. 2006; Junne 2010) to the fermentation medium.

Fermentation substrate is an important factor influencing the cost of butanol production (Qureshi and Blaschek, 2000; Kumar and Gayen, 2011). Lignocellulose is the most abundant renewable resource on the planet and it has great potential as a substrate for fermentation because of the un-competitiveness with food resources. Despite the advantages in sustainability and availability, commercial use of lignocellulose is still problematic. Due to the complexity of lignocellulosic materials, hydrolysis of hemicellulose and cellulose into five- and six-carbon sugars has to be carried out prior to, or concurrently with, the fermentation (Qureshi, 2007). Some reports on the corresponding ability of Clostridia strains are available in the scientific literature, but no systematic investigation has been carried out.

Present contribution regards the characterization of the ABE fermentation by *Clostridium acetobutylicum* DSM 792 adopting sugars representative for hydrolysis products of lignocellulosic biomass: hexoses (glucose and mannose) and pentoses (arabinose and xylose). Batch tests were characterized in terms of butanol and solvent yield and maximum solvent concentration. The conversion process was also interpreted on a metabolic level by comparison with glucose fermentations.

2. Materials and methods

2.1 Microorganism and medium

Clostridium acetobutylicum DSM 792 was supplied by DSMZ. Stock cultures were reactivated according to the DSMZ procedure. Reactivated cultures were stored at -80 °C. The thawed cells were inoculated into 12 mL synthetic medium containing glucose (30 g/L) and Yeast Extract (5 g/L) in 15 mL Hungate tubes (pre-cultures). Cells were grown under anaerobic conditions for 48 h at 37 °C, then they were transferred into fermentation bottles.

The standard fermentation medium consisted of 5 g/L YE supplemented to P2 stock solution: buffer) 0.25 g/L KH_2PO_4, 0.25 g/L K_2HPO_4, 2 g/L ammonium chloride; mineral) 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $MnSO_4 \cdot H_2O$, 0.01 g/L $FeSO_4 \cdot 7H_2O$ (Qureshi and Blaschek, 1999). The medium was sterilized in autoclave prior to the carbon addition. The carbon source (single sugar) was supplemented to the medium and filtered by sterilization. Four sugars - glucose, mannose, arabinose, and xylose - were investigated. The initial concentration of each sugar was set at 60 g/L. Some tests were also carried out with standard medium supplemented with 5 g/L $CaCO_3$.

2.2 Batch fermentation

Pyrex screw capped bottles (100 mL) containing 75 mL medium were used as fermenters. All experiments were carried out in fermenters at rest, at 37 °C, without pH control. The medium was

inoculated with 6.25 % (v/v) suspension of active growing pre-cultures. 3 mL of cultures were sampled periodically for cell/metabolites characterization.

2.3 Analytical procedures

pH was measured off-line in 1.5 mL samples by a pH-meter (Hanna Instruments). Analysis of culture samples was carried out after centrifugation at 10,000 rpm for 10 min. The liquid phase was characterized in terms of sugar and metabolite concentrations, total organic carbon (TOC), and total nitrogen (TN). Cell density was determined by measuring the absorbance at 600 nm (Cary-Varian mod. 50 scan UV-VIS spectrophotometer). Calibration tests indicated that the optical density is proportional to *C. acetobutylicum* dry mass under the operating conditions tested, in particular 1 OD_{600} corresponded to 0.4 g_{DM}/L. Elemental analysis of dry biomass was obtained by means of a C/H/N 2000 LECO® analyser. Sugar concentration was determined by high performance liquid chromatography (HPLC) using an Agilent 1100 system (Palo Alto, CA). The sugars were separated on a 8 μm Hi-Plex H, 30 cm 7.7 mm at room temperature and detected with a refractive index detector. Deionized water was used as mobile phase at a flow rate of 0.6 mL/min. A GC apparatus equipped with a FID, and outfitted with a capillary column poraplot Q (25 m x 0.32 mm) was used. Internal standard (hexanoic acid) was adopted to assess acids and alcohols and their concentrations. The TOC/TN was measured with a Shimadzu TOC 5000A analyzer. ABE (cell) yield was calculated as mass (dry) of ABE (cells) produced per mass unit of sugar converted and is expressed in g/g (g_{DM}/g).

3. Results and discussion

Investigations were aimed to assess the ability of *C. acetobutylicum* to ferment sugars typically present in the hydrolyzed fraction of lignocellulosic biomass. The tested sugars included glucose, mannose, arabinose, and xylose. The initial sugar concentration was 60 g/L (before inoculation) in all experiments. Effects of $CaCO_3$ supplement were also investigated.

C. acetobutylicum was able to convert all sugars into ABE. The performances assessed for each sugar changed in terms of both production rate and production yield. Tests carried out with glucose confirmed the high performance typically reported for this sugar: 12.5 g/L ABE produced within 160 h. The acidogenesis phase lasted less than 1 day. The Acetone:Butanol:Ethanol molar ratio was 3:6:1. The residual acid concentration at the end of fermentation was 1.26 g/L.

C. acetobutylicum had a similar growth pattern in all the fermentations except xylose where the microorganisms experienced reduced cell growth (data not shown).

Figure 1 reports the time-series of sugar concentration and ABE concentration measured during the fermentations of the four sugars. Figure 1A shows that *C. acetobutylicum* was able to convert the investigated sugars while the conversion degree decreased with the sugar species with the order glucose, mannose, arabinose, xylose. The maximum cell concentration measured during the fermentations - reported in Table 1 - changed according to sugar conversion degree. Regarding ABE production, three issues may be related to the sugar species: i) the final concentration of ABE; ii) the acidogenesis phase duration; and iii) the solvent production time (assumed as the time interval during which solvents are accumulated in the broth). The first and the last figure decreased with the sugar species with the order glucose, mannose, arabinose, xylose. The inverse order was observed in the second figure. The maximum butanol concentration measured at the end of the fermentation tests (Table 1) changed according to the ABE concentration scale.

Table 1: Fermentations of C. acetobutylicum. The reported data were the maximum value measured during the fermentation tests. Medium: standard

	Glucose	Mannose	Arabinose	Xylose
Cell (g_{DM}/L)	3.78	2.95	2.46	1.99
Cell yield (g_{DM}/g)	0.12	0.11	0.11	0.17
Residual acids (g/L)	1.27	1.57	2.48	3.39
Butanol (g/L)	7.87	7.1	4.7	2.52
ABE Yield (g/g)	0.39	0.42	0.38	0.3

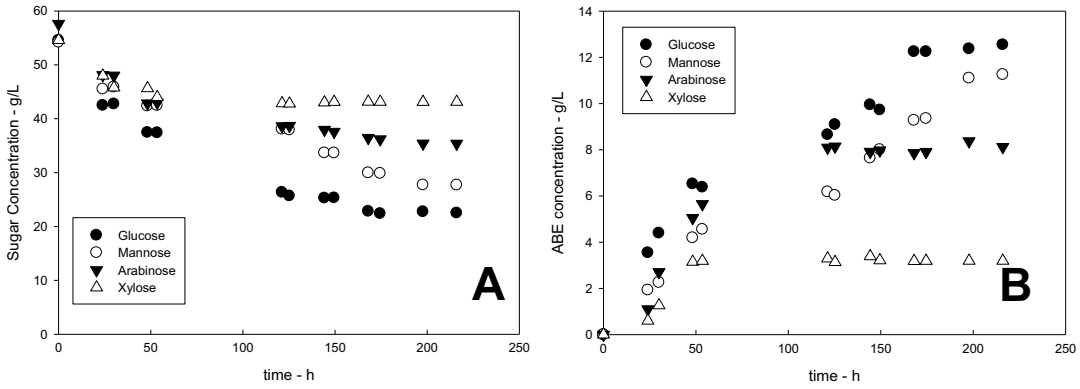

Figure 1: Production of ABE from individual sugar (60 g/L) using C. acetobutylicum. Medium: standard. A) Residual sugar concentrations vs. fermentation time. B) Total ABE concentration vs. fermentation time

Table 1 shows that the residual acid concentration and the ABE yield depended on the sugar species. In particular, the residual acid concentration was very high during tests with xylose and the ABE yield decreased significantly in these tests. These observations suggest a reduced capacity of *C. acetobutylicum* to uptake and recycle the acids when xylose – and in a less extent arabinose – was the carbon source.

Figure 2 and Table 2 report relevant data regarding fermentation tests carried out with standard medium supplemented with $CaCO_3$ (5 g/L) to assess the effects of $CaCO_3$ on the *C. acetobutylicum* performance. The main effects were:

- The sugar conversion degree of tests carried out with $CaCO_3$ was higher than that of tests carried out with the standard medium. Complete conversion was observed for glucose, mannose and arabinose;
- The sugar conversion rate (initial slope of the concentration vs. time) was definitively higher during the tests with $CaCO_3$ when compared with data from tests carried out with the standard medium. For glucose the ratio between the rates - $CaCO_3$ supplemented vs. standard – was about 5;
- Except for xylose, the maximum cell concentration appears not dependent on $CaCO_3$ presence. Regarding xylose, the maximum cell concentration measured during the fermentation in the $CaCO_3$ supplemented medium was about twofold compared to the standard medium;
- The acidogenesis phase in the $CaCO_3$ supplemented medium was longer than that observed during the fermentation in the standard medium;
- The maximum acids concentration was higher in $CaCO_3$ supplemented media than that measured during the fermentation in standard media. Nevertheless, the pH drop measured during acidogenesis was practically not dependent on the type of medium. It is likely that $CaCO_3$ modulated the dissociated-undissociated equilibrium of acetic-butyric acids;

Table 2: Fermentations of C. acetobutylicum. The reported data were the maximum value measured during the fermentation tests. Medium: standard supplemented with 5 g/L $CaCO_3$

	Glucose	Mannose	Arabinose	Xylose
Cell (g_{DM}/L)	3.91	3.03	2.66	4.28
Cell yield (g_{DM}/g)	0.07	0.05	0.05	0.10
Residual acids (g/L)	3.38	5.57	5.25	4.93
Butanol (g/L)	13.19	8.91	8.7	7.82
ABE Yield (g/g)	0.35	0.22	0.27	0.29

- The concentrations of both the ABE and the butanol measured at the end of the fermentation in $CaCO_3$ supplemented media were larger than that measured during the fermentation in standard medium. The molar butanol/ABE ratio was 0.61, 0.65, 0.54, and 0.65, respectively for glucose, mannose, arabinose, and xylose.

The reported results suggest that $CaCO_3$ promotes the butanol production without remarkable increase in cell concentration. The cell yield measured during the fermentation in $CaCO_3$ supplemented medium was about half the value measured during the fermentation in standard medium. For xylose, the beneficial effects apply to both cells and solvents. The promoting effects of $CaCO_3$ on butanol production may be due to an increase of stability of membrane proteins, provided the presence of bivalent ions (Ca^{2+}).

The enhancement of the sugar conversion with the $CaCO_3$ deserves some notes. Under batch conditions, the sugar is typically converted until the clostridial activity is inhibited by butanol titre (Grupe and Gottschalk, 1992; Qureshi and Blaschek, 2000;) – Figure 1b and Table 1. The unconverted substrate represents an economic loss in the process. However, this economic penalization appears to be removed for fermentations in $CaCO_3$ supplemented media: the sugar-substrate was practically completely converted (Figure 2, Table 2). An unconverted fraction of sugar is still found in fermentation of xylose even though $CaCO_3$ was supplemented. Altogether, $CaCO_3$ supplementation to the culture broth allows to both improve the exploitation of the feedstock and to enhance solvent production. Since the cost of the feedstock is one of the main items in the economic assessment of the ABE production (Qureshi and Blaschek, 2000), the complete conversion of the substrate appears a fundamental prerequisite for the success of the butanol production by fermentation. Therefore, the beneficial effects of $CaCO_3$ supplementation improve the economic advantage of the process.

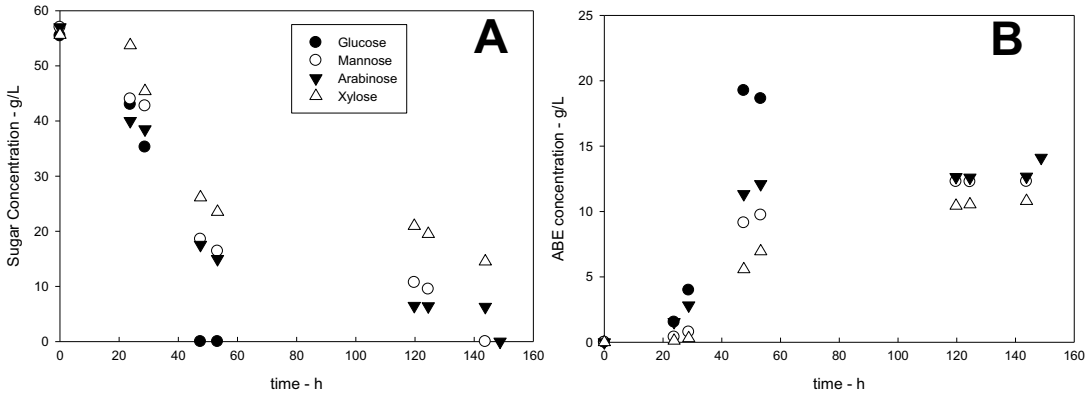

Figure 2: Production of ABE from individual sugar (60 g/L) using C. acetobutylicum. Medium: standard supplemented with 5 g/L $CaCO_3$. A) Residual sugar concentrations vs. fermentation time. B) Total ABE concentration vs. fermentation time

4. Final remarks

The results obtained from fermentation of different sugars showed that *C. acetobutylicum* is capable of utilizing products of lignocellulosic biomass hydrolysates for the production of solvents (ABE). Glucose was confirmed as the sugar characterized by the best performance. The fermentation performances of the other sugars decrease with the order mannose, arabinose, and xylose. The poor performance when using xylose can be explained on a metabolic level by various hypotheses:

- Additional energy demand from H+ dependent symport of xylose into cells;
- Inhibition or operon repression by other substrates or products (sugars/acids/solvents) (Ounine et al., 1985);
- Metabolic bottleneck in regeneration within the sugar conversion pathway by glyceraldehyde-3-P, which is a substrate for many other, competing reactions;

- Metabolic bottleneck in availability of transketolase, this enzyme catalyzes two reactions.

The $CaCO_3$ supplementation improves the fermentation performance in terms of both the conversion degree of the substrate and the final solvent concentration.

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