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Biobutanol Production from Hexose and Pentose Sugars

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The Acetone-Butanol-Ethanol (ABE) fermentation is receiving renewed interest as a way to upgrade renewable resources for the production of products with high added value as chemicals and fuels. Main pre-requisites of fermentation feedstocks are abundance and un-competitiveness with food sources and they are fulfilled by lignocellulosic biomass. This contribution reports about the characterization of the ABE fermentation by *Clostridium acetobutylicum* DSM 792 adopting sugars representative for hydrolysis products of lignocellulosic biomass: glucose, mannose, arabinose, and xylose. Batch fermentation tests with binary mixtures of sugars were performed to assess the possible crossed/coupled effects of the investigated sugars on the fermentation performances. The mass ratio of sugars in binary mixture tests was set at 1:1 and the total initial concentration was set at 60 g/L. The conversion process was characterized as a function of the time in terms of biomass, acids, and solvents concentrations as well as of pH and total organic compounds. The simultaneously fermentation of binary mixture of sugars enhances the conversion of the investigated sugars into butanol/solvents. The xylose fermentation appears to be improved when it is mixed with the investigated sugars.

1. Introduction

The interest in biofuel production is continuously increasing in the last decades for various reasons, among which: the exhaustion of worldwide oil deposits, the awareness in greenhouse gas emissions during combustion of fossil fuels, and the impacts of the anthropic activity on global warming. Butanol produced via ABE fermentation from renewable feedstocks using *Clostridium acetobutylicum* is a potential candidate as a biofuel. Main features of butanol are: low vapour pressure, blending with both gasoline and diesel at any fraction, energy density close to that of the gasoline, fuelled to current configuration of engines without any retrofitting, managing in the current infrastructures (Cascone, 2008).
Butanol is currently produced from the petroleum according to the Oxo process (reaction of alkenes with carbon monoxide and hydrogen). It may also be produced from renewable resources (biomass) by the acetone-butanol-ethanol (ABE) fermentation. The main actors of this fermentation process are the clostridia (saccharolytic butyric acid-producing bacteria). Several strains are capable to produce significant amounts of neutral solvents during the later stage of batch fermentations under selected 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 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 starts 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 the acidogenic cells – able to reproduce themselves - shift to the

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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 acidogenic phase, and one for the solventogenic phase (Jones and Woods, 1986).

The fermentation substrate plays a fundamental role in the cost of butanol production. The possibility to select feedstocks plentiful, inexpensive, and not edible is the success key for any process voted to produce biofuels from renewable resources (Qureshi and Blaschek, 2000; Kumar and Gayen, 2011; Raganati et al., 2013a). Lignocellulose is the most plentiful renewable resource on the planet, it is made of potential fermentable sugars, and it is not useful as food resources. Despite the advantages in sustainability and availability, processes based on lignocellulosic biomass are still far to be commercialized. Main obstacles for process commercialization are the pre-treatment steps and the fermentation of sugars mixtures produced during the pre-treatments. Apart from the mechanical comminution of the biomass, particular attention is paid at the hydrolysis of hemicellulose and cellulose that produces mixtures of five- and six-carbon sugars. Moreover, the fermentation of a such reach sugar mixtures is not an easy task because yield and kinetics related to the components of these mixtures range over wide spectra (Raganati et al., 2012; Qureshi, 2007). Typically, diauxic fermentation is observed when glucose and other sugars are present in the feedstock: the latter sugars are converted after glucose depletion. From an economic point of view, it would be preferred that pentose and hexose sugars are converted simultaneously (Ezeji et al., 2003). Although several scientific contributions are available in literature on the ability of Clostridia to produce ABE by conversion of sugars obtained from lignocellulosic biomass (Ezeji and Blaschek, 2008), an exhaustive systematic investigation has not been carried out yet.

A research program is active in Napoli regarding production of butanol by ABE fermentation (Napoli et al., 2010; Napoli et al, 2012; Raganati et al., 2013). First attempts to characterize the ABE production by *Clostridium acetobutylicum* DSM 792 fermentation of sugars typically present in lignocellulosic biomass hydrolysed have been reported (Raganati et al., 2012). Preliminary tests carried out according to the procedure reported by Napoli et al. (2009) were realized adopting single sugars - glucose, mannose, arabinose, and xylose - as carbon source to investigate the ability of the microorganism to convert them.

Previously, we examined the effect of each individual sugar on *C. acetobutylicum* growth and butanol production (Raganati et al., 2013b). These preliminary batch fermentation tests with single sugars 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.

Main results of the preliminary tests are reported in Table 1. It is evident that: i) *C. acetobutylicum* was able to convert the sugars typically present in lignocellulosic biomass hydrolysates into solvents (ABE); ii) glucose was confirmed as the sugar characterized by the best performance (13.2 g/L of butanol); iii) the fermentation performances of the other sugars decreased with the order mannose, arabinose and xylose.

Table 1 also shows that the residual acid concentration depended on the sugar species. In particular, the residual acid concentration was very high during tests with mannose, arabinose and xylose and the ABE yield decreased significantly in these tests with respect to the glucose fermentation. These observations suggest an higher capacity of *C. acetobutylicum* to uptake and recycle the acids when glucose was the carbon source.

Table 1: Fermentation tests of C. acetobutylicum. The reported data are the maximum value measured during the fermentation tests with single sugars (60 g/L) (after Raganati et al., 2012).

	hexoses		pentoses	
	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
Butanol (g/L)	13.19	8.91	8.7	7.82

This contribution moves a step further along this activity and reported results on the fermentation of mixtures of couple of sugars to assess the crossed/coupled effects of the investigated sugars on the fermentation performances. The total initial sugar concentration was set at 60 g/L. The mass ratio of sugars in binary mixture tests was set at 1:1. The conversion process was characterized as a function of the time in terms of biomass, acids, solvents concentrations and pH. Data were processed to assess sugar conversion and the kinetics of cell growth and of the ABE production. The yields of cells, acids and solvents on the carbon source were also assessed.

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 (YE) (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 fermentation medium consisted of 5 g/L YE and 5 g/L of $CaCO_3$ 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 $MgSO_4.7H_2O$, 0.01 g/L $MnSO_4.H_2O$, 0.01 g/L $FeSO_4.7H_2O$ (Napoli et al, 2011). The medium was sterilized in autoclave prior to the carbon addition. The carbon source (single sugar or couple of sugars) was supplemented to the medium and filtered by sterilization. Four sugars - glucose, mannose, arabinose, and xylose - were investigated. The overall initial concentration of each sugar was set at 60 g/L. The mass ratio of sugars in binary mixture tests was set at 1:1.

Tests were carried out with binary mixtures: Glucose/Mannose (GM), Glucose/Arabinose (GA), Glucose/Xylose (GX), Mannose/Arabinose (MA) and Arabinose/Xylose (AX).

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. No pH control was adopted in the present investigation.

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, Cell density was determined as absorbance at 600 nm by means of a spectrophotometer (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: 1 OD_{600} corresponds to 0.4 g_{DM}/L. 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.. 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

Fermentation tests have been carried out with binary mixtures of sugars. In particular, the fermentation tests have regarded the binary mixtures of sugars made of: glucose/mannose (GM), glucose/arabinose (GA), glucose/xylose (GX), mannose/arabinose (MA), and arabinose/xylose (AX). The nominal total initial sugar concentration has been set at 60 g/L and the mass ratio between the tested sugars was set at 1:1. Figures 1 and 2 report results of fermentation tests carried out adopting the mannose/arabinose (Fig. 1) and glucose/xylose (Fig. 2) mixtures as carbon sources. It is interesting to note that the microorganism simultaneously metabolized both the sugars. Although xylose has been converted slower than the other sugars, diauxic growth has not been observed. The conversion of sugars has been more extensive than that observed during the single sugar fermentation (Raganati et al., 2012). In particular, hexose depletion has been detected. It is interesting that xylose conversion has been about 35% during single sugar fermentation tests and has been about 60 % during the present tests.

The tests have been characterized in terms of the maximum value measured during the fermentations of cell concentration, residual acid concentration, butanol concentration, and solvent concentration. The cell yield has also been assessed.

Table 2 summarizes relevant data regarding the fermentation tests carried out with binary mixtures of sugars. The analysis of Figure 1 and 2 and Tables 1 and 2 points out the issues reported hereinafter.

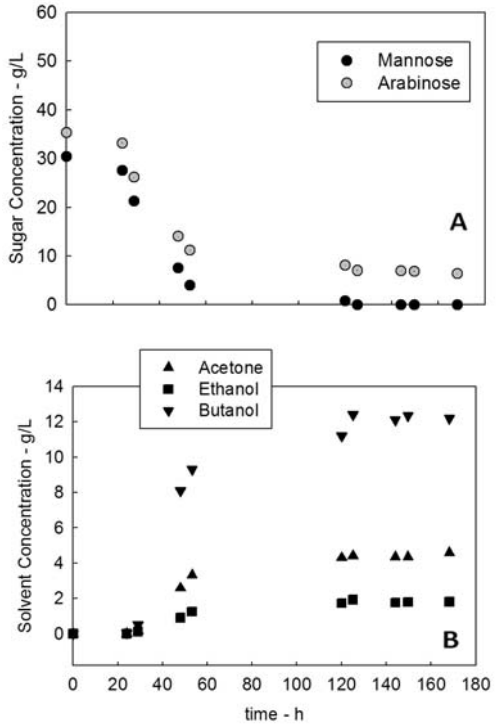

Figure 1: Time resolved concentration of mannose, arabinose, and solvents measured during C. acetobutylicum batch fermentation: A) residual sugar concentrations; B) solvent concentration.

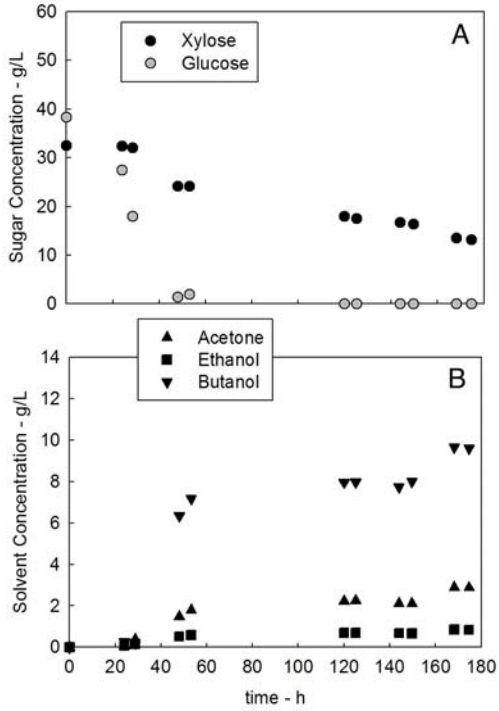

Figure 2: Time resolved concentration of glucose, xylose and solvents measured during C. acetobutylicum batch fermentation: A) residual sugar concentrations; B) solvent concentration.

- The high performances measured during the single sugar fermentation of glucose and mannose decrease remarkably when the glucose/mannose mixture has been adopted: e.g. the final butanol concentration (7.7 g/L) has been the lowest measured among the investigated binary mixtures.
- Fermentation tests carried out with mixtures including glucose (GA and GX) are characterized by low maximum concentration of butanol when compared with the fermentation tests carried out with glucose alone but high maximum concentration of butanol when compared to the tests carried out with the single pentoses (arabinose and xylose).
- The performances assessed for the fermentation tests carried out with the binary mixtures lacking in glucose (MA and AX) are markedly higher than those assessed during the tests carried out adopting the single sugar.
- From the point of view of cell production – the lost of substrate – the sugars binary mixtures behave as the single sugars except for the xylose. As a matter of fact, the xylose appears more convertible into solvents than into biomass.
- The best results were obtained for the couples including arabinose. In particular, the MA mixture has scored butanol concentration and yield (12.4 g/L and 0.21 g/g) similar to that obtained for glucose fermentation test (13.2 g/L and 0.24 g/g).

Table 2: Fermentations of C. acetobutylicum adopting binary sugar mixtures as carbon source. Nominal total initial concentration of sugars: 60 mg/L. The reported data are the maximum value measured during the tests.

	GM	GA	GX	MA	AX
Cell (g_{DM}/L)	2.75	3.29	2.35	4.18	3.18
Cell yield (g_{DM}/g)	0.06	0.05	0.04	0.07	0.05
Residual acids (g/L)	5.25	4.02	4.97	4.29	5.60
Butanol (g/L)	7.67	11.5	9.65	12.4	11.4

Reported results suggested that *C. acetobutylicum* may grow by converting simultaneously hexose and pentose sugars. The fermentation performances of glucose got worse when it has been mixed with the other sugars investigated, whatever the class of the second sugar. The other three sugars, on the contrary, benefited of the combination with a second sugar.
As a general result, the butanol/ABE selectivity assessed for the binary mixtures ranges between 0.60 and 0.73, higher than that assessed for the single sugar tests.

4. Final remarks

The *C. acetobutylicum* fermentation adopting binary solutions of sugars typically present in the lignocellulosic biomass hydrolysates has been successfully investigated. The aim of this work is to assess the possible crossed/coupled effects of the investigated sugars on the fermentation performances. In particular, the fermentation tests regarded the binary mixtures of sugars made of: glucose/mannose (GM), glucose/arabinose (GA), glucose/xylose (GX), mannose/arabinose (MA), and arabinose/xylose (AX). Tests have pointed out that *C. acetobutylicum* is able to simultaneously metabolize the investigated sugars (glucose, mannose, arabinose, and xylose) and no diauxic growth has been observed.
Except for glucose, the maximum concentration of butanol measured during tests with the binary mixtures was higher than that assessed during the test with single sugars. It was close to that assessed for glucose fermentation. In particular, the best results were obtained for the couples including arabinose: the MA mixture has scored butanol concentration and yield (12.4 g/L and 0.21 g/g) similar to that obtained for glucose fermentation test (13.2 g/L and 0.24 g/g).

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