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Comparison of strategies to overcome the inhibitory effects in high-gravity fermentation of lignocellulosic hydrolysates

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ABSTRACT

High-gravity (HG) technology aims at generating final ethanol concentrations above 50 kg m^{-3} in order to reduce the cost of the distillation step. The generation of higher amounts of inhibitors during the pretreatment step is one of the challenges that accompany the increase in initial dry matter. Detoxification of spruce hydrolysate, adaptation of the cells before fermentation, supplementation with nutrients, and washing of solids were the strategies compared in this study. They represent different approaches to cope with the inhibitory effects, and we compared their efficiencies using a thermotolerant strain of *Saccharomyces cerevisiae* at temperatures from 30 °C up to 40 °C.

The dilute acid-pretreated spruce used as substrate in this study was not fermentable under HG conditions (200 g kg^{-1} water-insoluble solids) when no improvement method was applied. In HG simultaneous saccharification and fermentation at 30 °C combined with a 24 h pre-hydrolysis step, the detoxification of pretreated spruce with reducing agent ($Na_2S_2O_4$) gave the best result with an ethanol yield of 57% (on total sugars) of the maximum theoretical and a volumetric productivity of 1.58 g dm^{-3} h^{-1}. In HG separate hydrolysis and fermentation, nutrients supplementation gave better final ethanol yields than detoxification of the material, reaching an ethanol yield of about 60% of the theoretical (on total sugars). The results obtained, showed an increase in severity of inhibitory effects with temperature increase. Improved cell viability was observed when detoxified material was used and also when yeast extract addition was coupled with adaptation of the cells to the hydrolysate.

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1. Introduction

High-gravity (HG) technology—i.e. operation of processes with high solid loadings of lignocellulosic materials—is used to generate final ethanol concentrations of more than 40–50 kg m^{-3} [1]. To achieve this final ethanol concentration, the substrate concentration used in the process must be significantly higher than the levels commonly used today. Not only will the costs associated with distillation and evaporation be reduced, but also the cost of fermentation. Increasing the substrate concentration results in reduced water

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consumption, which reduces the energy demand for distillation and evaporation, provided that the ethanol yield is maintained at a high level [2].

In order to obtain high yields under HG conditions, however, many challenges must be faced. Cellulosic raw materials are hygroscopic and they have lower density than water. With concentrations of total solids exceeding 150 g kg^{-1}, cellulosic slurries become progressively more paste-like. Thus, they become viscous and difficult to handle. Furthermore, high loading of solids in the pretreatment step means higher amounts of inhibitors in the pretreated material and consequently higher concentrations of these in the fermentation medium. It has been shown that this is particularly important when hydrothermal or steam-explosion pretreatments (in the presence of acids) are used, where, even at low loading of solids, high concentrations of inhibitors are generated [3]. The kinds and the amounts of different inhibitors generated depend on the raw material used and on the conditions of the pretreatment. The degradation products can be divided into three groups according to their origin: furan derivatives, weak acids, and phenolic compounds derived from sugars, hemicellulose, and lignin [3]. Their inhibition mechanism is not only based on the inhibitory effect caused by each compound individually, but also on their synergistic action [4]. The effects of inhibitors on cell growth and on ethanol yields and productivities not only depend on the presence of the inhibitors themselves, but also on other conditions that prevail during fermentation [5].

To overcome the challenge of high concentrations of inhibitory compounds in the fermentation broth under HG conditions, different strategies have been suggested [6]. With some approaches, the aim is to reduce the generation of toxic compounds by modifying the conditions of the pretreatment itself [7,8]. Evolutionary engineering focuses on improving the characteristics of the fermenting microorganism. The strains (or the populations) that are generated through evolution of the fermenting yeast in the presence of inhibitors before the process have better ability to handle the inhibitory lignocellulosic hydrolysates, in many cases giving improved fermentative performance compared to the corresponding unevolved strain [9]. Supplementation of the fermentation medium with nutrients has also proven to be a successful strategy to address inhibitory issues, enhancing the innate tolerance of the yeast to the inhibitors [10,11]. Alternatively, detoxification strategies focus on the removal or reduction of inhibitors in order to increase the fermentative performance of the microorganism in use. Removal of inhibitors from the hydrolysate can be done by various methods such as solvent extraction, anion exchange, overliming, use of zeolites or laccase enzymes [12], or addition of reducing agents [13].

During the past 3 years, some scientific reports have been published on possible ways to address the inhibitory issues during fermentation under HG conditions [14–17]. However, each one has focused on only one approach (cells adaptation or detoxification of the material) to enhance the fermentability of lignocellulosic materials. In addition, various raw materials at different solids loadings have been used in these studies. In the present study, we compared different strategies to minimize the deleterious effects of the inhibitory compounds that occur during fermentation of lignocellulosic feedstocks under HG conditions. Dilute acid steam-pretreated spruce slurry was chosen as the substrate, due to its high toxicity for yeast cells [9,17]. The influence on ethanol yield and productivity of using separate hydrolysis and fermentation and of applying simultaneous saccharification and fermentation was studied to differentiate between the inhibitory effects using different fermentation conditions. The methods used were optimized regarding various process parameters. To the best of our knowledge, this is the first time that such a comparative study has been performed using high-gravity conditions.

2. Materials and methods

2.1. Microorganism

The industrial *Saccharomyces cerevisiae* strain Thermosacc© (Lallemand Biofuels & Distilled Spirits, USA) was used in this study. Thermosacc© cannot utilise C5 sugars. The strain was stored as glycerol suspensions (40 cm^3glycerol/100 cm^3 of suspension) in cryo-vials at −80 °C. Volumes of 100 mm^3 from the vials were used to inoculate precultures.

2.2. Materials, media, and chemicals

Unless otherwise stated, all chemicals used in the project were of analytical grade and were purchased from Sigma Aldrich (Sweden). Yeast extract was purchased from Becton Dickinson (Sweden).

Spruce (*Picea abies*) slurry was used as the substrate in all simultaneous saccharification and fermentation (combined with a pre-hydrolysis step, P-SSF) experiments and separate hydrolysis and fermentation (SHF) experiments. In the P-SSF experiments, a pre-hydrolysis step was added (24 h); thus, we will refer to this configuration as P-SSF throughout this study. Spruce slurry was provided by SEKAB E-Technology AB (Örnsköldsvik, Sweden) and it was prepared from spruce wood chips pretreated with dilute acid (SO_2 in water). The pretreatment temperature in the reactor was 210–215 °C, the average reactor residence time was 5–7 min, and SO_2 (g) was used in a final concentration of 20 g dm^{-3} of dry matter feedstock. The pH was set at 5.0 using 1 mol L^{-1} NaOH and nutrients were added according to the experimental design. Filtrate of the spruce slurry was used in all screening fermentations and optimization experiments as well as for the adaptation of the cells. For preparation of the spruce hydrolysate, the slurry was centrifuged and the supernatant was filtered using filters of 0.2 mm^3 pore size. The analysis of the material was done according to the NREL protocol for the determination of structural carbohydrates and lignin in biomass [18]. The compositions of the slurry and the filtrate are given in Table 1.

The spruce hydrolysate (the filtrate from spruce slurry) and all other liquid media were sterilized by passing through filters of pore size 0.2 μm, whereas the slurries for P-SSF and SHF experiments were not sterilized. Flasks, syringes, and other sampling equipment were sterilized by autoclavation (121 °C for 0.5 h).

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