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Bioethanol production from steam-pretreated corn stover through an isomerase mediated process

Research Paper

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Agricultural by-products such as corn stover are considered strategic raw materials for the production of second-generation bioethanol from renewable and non-food sources. This paper describes the conversion of steam-pretreated corn stover to ethanol utilising a multi-step process including enzymatic hydrolysis, isomerisation, and fermentation of mixed hydrolysates with native *Saccharomyces cerevisiae*. An immobilised isomerase enzyme was used for the xylose isomerisation along with high concentrations of *S. cerevisiae*. The objective was to assess the extent of simultaneity of the various conversion steps, through a detailed analysis of process time courses, and to test this process scheme for the conversion of lignocellulosic hydrolysates containing several inhibitors of the isomerase enzyme (e.g. metal ions, xylitol and glycerol).

The process was tested on two types of hydrolysate after acid-catalysed steam pretreatment: (a) the water soluble fraction (WSF) in which xylose was the largest carbon source and (b) the entire slurry, containing both cellulose and hemicellulose carbohydrates, in which glucose predominated.

The results indicated that the ethanol concentration rose when the inoculum concentration was increased in the range 10–75 g/L. However, when xylose was the largest carbon source, the metabolic yields were higher than 0.51 $g_{ethanol}/g_{consumed\ sugars}$ probably due to the use of yeast internal cellular resources. This phenomenon was not observed in the fermentation of mixed hydrolysates obtained from the entire pretreated product and in which glucose was the largest carbon source. The ethanol yield from biomass suspensions with dry matter (DM) concentrations of 11–12% (w/v) was 70% based on total sugars (glucose, xylose, galactose). The results suggest that xylulose uptake was more effective in mixed hydrolysates containing glucose levels similar to, or higher than, xylose.

Analysis of the factors that limit isomerase activity in lignocellulosic hydrolysates excluded any inhibition due to residual calcium ions after the detoxification of the hemicellulose hydrolysates with $Ca(OH)_2$. By contrast, most of the enzyme activity ceased during the fermentation of the entire slurry after steam explosion, probably due to synergistic inhibition effects of various fermentation co-products.

Introduction

Since the 1980s, the use of bioethanol as a transport fuel has been considered to be a suitable replacement for oil based fuels. Drivers for this include the volatility of the oil market and the need for a degree of energy independence. Bioethanol can be produced, through suitable pretreatment and fermentation processes, from several dedicated crops and agricultural residues. These include lignocellulosic feedstocks, such as wheat straw and corn stover

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1871-6784/$ - see front matter http://dx.doi.org/10.1016/j.nbt.2013.12.003

(namely stalks and leaves), that are among the most abundant resources on the planet for production of so-called 'second-generation biofuels' and biobased products. Unlike first generation biofuels that are derived from the edible portion of plants, these advanced biofuels do not compete with food crops and produce fewer greenhouse gas (GHG) emissions.

Agricultural straws and stovers contain cellulose and a significant amount of xylan. This requires that, for optimal ethanol production, all the available carbohydrates are efficiently converted. In particular, the conversion of xylose to ethanol leads to an increase of the overall yield and could make the process more economically viable [1–4]. The native strains of *Saccharomyces cerevisiae* are widely accepted as having a low, if any, ability to utilise xylose as a carbon source for ethanol production [5–7]. Some other yeasts, such as *Pachysolen tannophilus*, *Pichia stipitis*, *Candida shehatae*, have demonstrated a good ability to convert xylose into ethanol [8–11]. However, their fermentation performance crucially depends on the maintenance of microaerophilic conditions during the process [11–13]. Furthermore, xylose uptake and metabolism in mixed hydrolysates could be limited by glucose (diauxic shift) [14].

Remarkable breakthroughs have been achieved by using engineered microrganisms [15–18]. In some cases, the overexpression of key enzymes also improved yeast resistance in lignocellulosic hydrolysates [19]. Another viable option for xylose fermentation is the intermediate conversion into the corresponding ketose, namely xylulose, which can be metabolised by *S. cerevisiae* through the pentose phosphate pathway (PPP) [3,20]. This can be performed by a glucose isomerase (GI) (D-xylose ketol-isomerase; EC 5.3.1.5) able to catalyse the reversible isomerisation of D-glucose and D-xylose to D-fructose and D-xylulose, respectively. Previous investigations demonstrated that this process is promoted by the addition of borate ions, which shift the equilibrium toward increased xylulose formation [21]. In fact, they bind more tightly to xylulose than to xylose and effectively reduce product concentration.

The GI enzyme is already used in an industrial application for the conversion of glucose into highly concentrated fructose syrups [22]. The possibility of producing ethanol through isomerase-mediated fermentation of xylose is not new [23–25]. Since the early investigations, the major difficulties encountered have been that many xylose isomerases work at high temperature (60–80°C) and high pH (7.0–9.0) in contrast to the optimal conditions for yeasts (28–37°C and pH 4.8–5) [26,27]. Several approaches have been explored to run the two processes simultaneously. Chandrakant and Bisaria demonstrated the possibility of overcoming this limitation by using *S. cerevisiae* and a compatible xylose isomerase from *Candida boidinii* having a pH optimum of 4.5–5.0 and temperature range of 30–35°C [28]. More recently, a renewed interest in this approach has demonstrated some interesting results [29–31]. In particular, Rao et al. [29] described the possibility of using co-immobilised isomerase and urease in the presence of urea in the external medium to lower the isomerase working pH to 5.0. This strategy coupled with the use of concentrated inocula (up to 200 g/L) demonstrated the feasibility of using native strains to ferment synthetic sugar mixtures [31].

Methods examined to overcome the need for different conditions in a multi-step processing include the use of hybrid processes (i.e. semi-simultaneous), the choice of compromise conditions, and/or a gradual change of process parameters. In this paper, the fermentation of corn stover-derived carbohydrates is investigated by developing a process scheme consisting of the sequential addition of the enzymes and microrganism and in the choice of compromise conditions for temperature and pH. The aim was to assess how close the process could be brought to a simultaneous conversion.

Apart from optimising the processing of sugars, the final ethanol yields also depend on the hydrolysate composition. In this regard, two aspects were evaluated: the inhibitory effect on the isomerase of some common components in lignocellulosic hydrolysates and the xylulose metabolism as a function of the hydrolysates sugar composition. A stepwise approach was followed. First, various process configurations were tested by using synthetic solutions containing only xylose to explore both the combined effect of borate addition and process strategy on the final ethanol yields. Second, the process was tested on lignocellulosic hydrolysates of corn stover and, in particular, on the water soluble fraction (WSF) after steam explosion pretreatment. Detoxification of the hydrolysates by addition of calcium hydroxide (Ca-overliming) was also considered before fermentation. At the same time, the concentrations of residual Ca^{2+} and Mg^{2+} ions were monitored and the relevant variations associated to the isomerase activity deduced from the experimental findings. Finally, the bioconversion of the entire slurry, after steam explosion pretreatment, was investigated by selecting the best process configuration and the biocatalyst dosages assessed within this series of experiments.

Materials and methods

Corn stover pretreatment and hydrolysis

Corn stover was harvested from the north of Italy. The dry matter (DM) content of the raw material was 90%. Ground corn stover (0.2 mm) was analysed for carbohydrates, lignin and ash content by the following standard methods: carbohydrates and lignin by the Klason procedure (modified TAPPI T-13 m-54 and ASTM D1106), and ash by ASTM D1102. The detailed procedures are described elsewhere [32]. The raw material contained (%): 37.6 ± 1.10 glucan, 26.7 ± 0.80 xylan, 2.11 ± 0.09 arabinan, 0.86 ± 0.02 galactan, 15.5 ± 0.40 Klason lignin, 5.05 ± 0.12% total ash, and 6.60 ± 0.50% extractives. An acid-catalysed steam explosion pretreatment at mild conditions (190°C for 5 min) was chosen to avoid severe hemicellulose degradation to furan compounds and small chain acids while ensuring adequate cellulose fiberisation [33,34]. Before the steam pretreatment, corn stover was ground into smaller particles, the distribution of which was classified using a sieve shaker. The data showed that the majority of the corn stover substrates consisted of particles within a size range of 4–7 mm. 0.7 kg of raw material was soaked in 10 kg of dilute sulfuric acid (0.07 M) at room temperature for 15 min and then pressed to remove the excess liquid according to the procedure previously reported [34]. The DM content of the impregnated biomass was in the range 32–34% corresponding to an acid loading of approximately 1.3 wt% (based on moisture content).

Biomass pretreatment was carried out in a steam explosion batch digester (from Staketech) processing 1 kg of biomass per cycle. The pretreated product had a slimy consistency and contained both hexose and pentose sugars. After the acid-catalysed