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# Continuous recycling of enzymes during production of lignocellulosic bioethanol in demonstration scale

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## HIGHLIGHTS

- Results from continuous experiments in demonstration scale for a total of 16 days.
- Reuse of enzymes is possible through recycling fermentation broth.
- Recycling fermentation broth can increase ethanol concentration with lower dry matter.

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## ABSTRACT

Recycling of enzymes in production of lignocellulosic bioethanol has been tried for more than 30 years. So far, the successes have been few and the experiments have been carried out at conditions far from those in an industrially feasible process. Here we have tested continuous enzyme recycling at demonstration scale using industrial process conditions (high dry matter content and low enzyme dosage) for a period of eight days. The experiment was performed at the Inbicon demonstration plant (Kalundborg, Denmark) capable of converting four tonnes of wheat straw per hour. 20% of the fermentation broth was recycled to the hydrolysis reactor while enzyme dosage was reduced by 5%. The results demonstrate that recycling enzymes by this method can reduce overall enzyme consumption and may also increase the ethanol concentrations in the fermentation broth. Our results further show that recycling fermentation broth also opens up the possibility of lowering the dry matter content in hydrolysis and fermentation while still maintaining high ethanol concentrations.



## 1. Introduction

The recycling of enzymes in production of bioethanol from lignocellulosic biomass aiming at reducing enzyme consumption and thereby production costs have been investigated for many years and by many different approaches. These typically involve either contact between the lignin-rich residue after hydrolysis/fermentation and fresh cellulose containing substrate [1–5], different methods for recycling process liquids or combinations thereof [6]. Liquid recycling is furthermore often combined with other process steps *e.g.* pH adjustment [7–11] or alkaline elution [12] to desorb enzymes prior to separation or membrane filtration to concentrate the enzymes and reduce the amount of water to be recycled [13,14]. Previously published experiments with enzyme recycling show that the cellulolytic activity remaining after hydrolysis and fermentation is found both free in solution and adsorbed to the residual solids [15] – often most of the cellulases are adsorbed to the residual solids [3,4]. The amount of adsorbed activity depends on the type of biomass, the type of pretreatment [16], and the type of enzyme [17]. Therefore, recycling methods need to be adapted to the individual pretreatment process and should include either recycling of both liquid and solids or a method for protein desorption (*e.g.* one of the methods mentioned earlier).

Commercial cellulase preparations for hydrolysis of pretreated biomass contain different enzyme activities to ensure efficient

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hydrolysis. These include cellulases (endo- and exoglucanases), hemicellulases, β-glucosidases, oxidative enzymes, esterases and several others [18–21]. This mixture of different activities complicates the recycling process since each enzyme behaves differently with regard to stability during the process and adsorption to cellulose and/or lignin.

A key challenge in recycling of cellulase preparations is the rather variable adsorption of cellulases and β-glucosidases to the matrix of lignocellulosic biomass [4,15,17,22,23]. Therefore, from a point of process simplicity, the best results for enzyme recycling may be obtained when the whole fermentation broth is reintroduced to the hydrolysis step.

With regard to enzyme recycling, adsorption to the biomass during the process of cellulose hydrolysis and the irreversible loss of some of the cellulase activities are both major challenges. This can be due to a number of factors such as denaturation from the residence time at elevated temperature [23], enzyme precipitation [24], shear stress (from agitators, flow and pumps) [25–30] and/or denaturation from contact with the air–liquid interphase. We have previously shown that depending on the cellulase preparation, residence time, and temperature, 35–75% of the cellulase activity is irreversibly lost when the cellulases are incubated in buffer without substrate [23]. However, we lack knowledge on the levels of individual cellulase activities in a continuous industrial process, where denaturation, inhibition, and lignin build-up might reduce the enzyme recycling potential severely.

Recently, Weiss et al. [31] have shown that significant amounts of cellulase activity can be recycled through recycling the residual solids making it possible to reduce the enzyme dosage by 30% and still reach the same glucose yields. Tu et al. [32] have optimised a method for enzyme desorption from the residual solids using 0.5% Tween 80 at app. 44 °C and pH 5.3 resulting in a cellulose conversion after three successive rounds of recycling of 88%. However, most of the published experiments have been carried out at conditions that are not feasible in an industrial process for production of advanced bioethanol. A major problem is typically that the hydrolysis have been carried out at low dry matter content (2–15%), high enzyme loadings and with advanced setups for separation and enzyme recovery, which are not possible or realistic to implement on a larger scale.

In 2009, DONG Energy started operating the Inbicon demonstration plant in Kalundborg (Denmark). The plant is capable of processing four tonnes of wheat straw per hour and wheat straw is currently converted into three products; ethanol, a C5 molasses (for production of biogas, animal feed or bioethanol), and solid lignin biofuel (for production of green electricity and heat). A thorough description of the demonstration plant has been published previously [33].

The aim of this work was to test enzyme recycling by recycling fermentation broth in the Inbicon demonstration plant at a dry matter above 20% content and in a continuous production run over an extended period. We have taken an approach of process simplicity when testing enzyme recycling at demonstration scale by continuously recycling 20% of the fermentation broth to the first hydrolysis stage. According to our previous findings [23], the fermentation broth was the most promising stream to recycled as it had a higher activity than the distillation broth while containing less inhibitory compounds than the hydrolysis mash. The experimental setup can be seen in Fig. 1. The demonstration scale experiment was carried out in two parts each app. eight days – first a reference phase without enzyme recycling followed by a recycling phase where the fermentation broth produced in the reference run was recycled to the hydrolysis. During both phases cellulose conversion, ethanol yield, and the activities of Cel7A (cellobiohydrolase I), Cel7B (endoglucanase I), and β-glucosidase were monitored.

## 2. Results and discussion

### 2.1. Continuous enzyme recycling in demonstration scale

During the recycling test, the level of recycled fermentation broth was monitored by measuring the ethanol concentration in the hydrolysis mash and the recycled fermentation broth; the ethanol concentration could then be used as an internal standard to calculate the degree of recycling (according to Eq. (1)). Data from the experiment confirmed that an overall recycling degree of 18% (0.18) was achieved.

In the recycling run, the enzyme loading was reduced by 5% compared to the reference run. The 5% reduction was chosen based on our previous laboratory scale findings of low enzyme stability combined with the level of enzyme activity generally observed in samples of fermentation broth from pilot- and demonstration scale [23]. The previous studies yielded total recoveries of cellulase activity of 59% and 41% depending on enzyme preparation in the complete fermentation broth [23]. However, in reality it is not possible to recycle the complete broth (as there would be no output from the process) and therefore based on practical considerations it was decided to recycle 20% of the fermentation broth. This amount should correspond roughly to recycling of 5% of initial activity and thereby enable a 5% reduction of initial enzyme loading. Furthermore, the addition of water to the pretreated biomass was reduced in the recycling run compared to the reference run to compensate for the addition of fermentation broth and keep the dry matter content identical in both reference and recycling run (app. 20.5%). A 5% reduction in enzyme level is a minor change and demands great attention to measurements as well as process parameters.

The conversion of cellulose during hydrolysis for the reference and recycling runs can be seen in Fig. 2 (the conversions are calculated according to Eq. (2)). The data for the two runs are fitted to a one-phase association model demonstrating statistical differences between the two ($p$-value less than 0.0001). However, the models of the experimental data showed that similar conversions could be achieved in the reference and recycling runs, when the mean residence time in the recycling run is extended, although using 5% less enzyme in the recycling trial. According to the two models, the mean residence time should be extended from app 95 h in the reference run to app 130 h in the recycling run to obtain 70% cellulose conversion.

The slower hydrolysis observed in the recycling run may have been caused by the presence of enzyme inhibitors in the recycled fermentation broth. These include ethanol [34–36], xylose [37], degradation products from the pretreatment [38]. The fact that the hydrolysis levels of around 70% can also indicate that the remaining 30% cellulose is rather recalcitrant and therefore difficult to degrade as indicated by Fig. 2. This recalcitrance could either be due to the cellulose structure, i.e. crystallinity, or the increasing concentration of lignin hindering the accessibility of the cellulases to the cellulose [16,39].

However, even though the cellulose conversion is lower in the recycling run than in the reference run the final ethanol concentrations are identical due to the ethanol in the recycled fermentation broth. If the hydrolysis is extended to obtain identical cellulose conversions, this will lead to an increased ethanol concentration in the fermentation broth, which will reduce energy consumption for distillation.

### 2.2. Recovery of enzyme activity in demonstration scale

During the experiment we tested if the adsorbed enzymes in the fermentation broth could be desorbed by alkaline wash using the method previously described by Rodrigues et al. [12]. The method was able to desorb minor amounts of Cel7B whereas no