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ORIGINAL RESEARCH PAPER

Cellulose hydrolysis by immobilized *Trichoderma reesei* cellulase

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Abstract Cellulose hydrolysis by immobilized *Trichoderma reesei* cellulase in the presence of a low viscosity ionic liquid, 1-ethyl-3-methylimidazolium diethyl phosphate (EMIM-DEP), was investigated. Preparation of the carrier-free immobilized cellulase was optimized with respect to concentration of the cross-linker and the type of precipitant. The addition of 2% (v/v) EMIM-DEP during hydrolysis gave an initial reaction rate 2.7 times higher than the hydrolysis rate with no ionic liquid. The initial yield after 2 h was 0.7 g glucose/g cellulose, and the carrier-free immobilized cellulase (CFIC) was effectively re-used five times.

Keywords Bioethanol · Carrier-free immobilized cellulase · Cellulose · Ionic liquid

Introduction

Ethanol derived from cellulosic material is a ‘green’ alternative to fossil-based fuels since it can be produced economically, has a high octane number and reduces green house gas emissions (Lee et al. 1991). Currently four billion US gallons of ethanol in the United States is produced annually, mainly from corn. Researchers are split on whether ethanol from corn is energy efficient. According to Pimentel and Patzek (2005) “Ethanol production using corn grain requires 29% more fossil energy than the ethanol fuel produced.” Wang et al. (2005) dispute this and state that it takes 0.74 BTU of fossil fuel to create 1 BTU of ethanol fuel, compared with a ratio of 1.23 BTUs to 1 BTU for gasoline or 66% more than ethanol. The conclusions of Wang et al. (2005) have largely been corroborated by Farrell et al. (2006). According to them, “current corn ethanol technologies are much less petroleum-intensive than gasoline but have greenhouse gas emissions similar to those of gasoline.” The authors however state that ethanol from cellulosic sources is vital to its large-scale use as a fuel. Hammerschlag (2006) compared data from ten different studies and used a parameter, r_E, defined as the total product energy divided by nonrenewable energy input to its manufacture. Thus, $r_E > 1$ indicates that the ethanol has captured some renewable energy and $r_E > 0.76$ indicates that it consumes less nonrenewable energy in its manufacture than gasoline. The corn ethanol studies showed r_E in the range $0.84 \leq r_E \leq 1.65$, and three of the cellulosic ethanol studies indicated a range of $4.40 \leq r_E \leq 6.61$. Therefore, ethanol from cellulosic material is a better form of ethanol than corn-based ethanol. The major steps in producing ethanol from cellulosic sources are pretreatment, enzymatic hydrolysis and fermentation.

Pretreatment of cellulose is a necessary step in the production of ethanol from cellulosic material since it

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makes the recalcitrant cellulosic biomass more accessible to enzymatic hydrolysis (Zhang and Lynd 2004). Chemical, hydrothermal and physical pretreatment processes have been used with varying degrees of success. An emerging chemical pretreatment step is the use of ionic liquids (Dadi et al. 2006). Ionic liquids are organic salts that are liquid at room temperatures. The anions in ionic liquids bond with cellulose at high temperatures, dissolving the cellulose (Youngs et al. 2007). This results in better enzymatic hydrolysis. However, the presence of high concentrations of some ionic liquids results in the inactivity of the enzyme (Zhao et al. 2009; Turner et al. 2003). Regenerating the cellulose and recovering the ionic liquid has been used to alleviate this problem (Dadi et al. 2006). The regeneration step can be eliminated if ionic liquids that do not denature cellulase are used. It has been found that a 1:4 (v/v) mixture of 1-ethyl-3-methylimidazolium diethylphosphate with water is effective in increasing hydrolysis yields (Kamiya et al. 2008). Immobilization of enzymes, in addition to imparting greater stability to the enzyme, is also an important step for enzymatic hydrolysis of cellulose to be cost effective.

In this work, immobilized cellulase is used in conjunction with very low concentrations of 1-ethyl-3-methylimidazolium diethyl phosphate. The immobilization is achieved using a carrier-free method, which minimizes any dilution of catalytic activity (Sheldon 2007).

Materials and methods

Materials

Cellulase from *Trichoderma reesei* and microcrystalline cellulose were purchased from Sigma-Aldrich (St. Louis, USA). 1-Ethyl-3-methylimidazolium diethyl phosphate was purchased from Alfa Aesar. Roche Accu-chek Active glucose meter and test strips were used for measuring glucose concentration.

Methods

Immobilization

Nine ml of chilled acetone at 10°C were added to a 25 ml glass vial with a magnetic stirrer. 150 U of cellulase enzyme were dissolved in 1 ml of 0.1 M citric acid/phosphate buffer at the isoelectric point (5.3) and the solution added to the chilled acetone. Glutaraldehyde was then added dropwise to give 5 mM. The mixture was kept at 10°C for 2.5 h with gentle stirring. Five ml buffer was then added and the mixture centrifuged at 600×*g* for 10 min. The supernatant was decanted and the pellet washed three times with buffer. The final, washed, carrier-free immobilized cellulase was kept overnight in 1 ml buffer at 4°C. To evaluate the reusability of carrier-free immobilized cellulase (CFIC), the size was limited to less than 20 μm. Separation was achieved via filtration with Nitex 20 μm nylon mesh.

Enzymatic hydrolysis

Cellulose 50 mg, 120 U immobilized enzyme and 10 ml buffer were added to the reactor. Samples were withdrawn periodically and the glucose concentration measured using a glucose meter. The immobilized enzyme was recovered after 24 h and reused. When using 1-ethyl-3-methylimidazolium diethyl phosphate (EMIM-DEP) as a pretreatment step, 0.2 ml of EMIM-DEP was added to 50 mg cellulose and the mixture heated for 10 min at 105°C. Ten ml 0.1 M phosphate buffer was then added and vigorously stirred at 700 rpm for 0.5 h. The immobilized enzyme (120 U) was then added and the stirring speed was decreased to 325 rpm. Samples were withdrawn periodically and the glucose concentration measured using a glucose meter. The yield of glucose was calculated as the concentration of glucose divided by the initial concentration of cellulose. Each experiment was performed twice at pH 5.0 and 45°C and the average yield was calculated.

Results and discussion

Immobilized enzyme

The preparation of the immobilized enzyme was optimized by investigating the effect of concentration of cross-linker as well as the type of precipitant. Four precipitants were investigated: methanol, n-propanol, acetone and dimethyl ether. As shown in Fig. 1, the immobilized enzyme prepared with acetone retained the highest activity with a yield of 0.13 g glucose/g

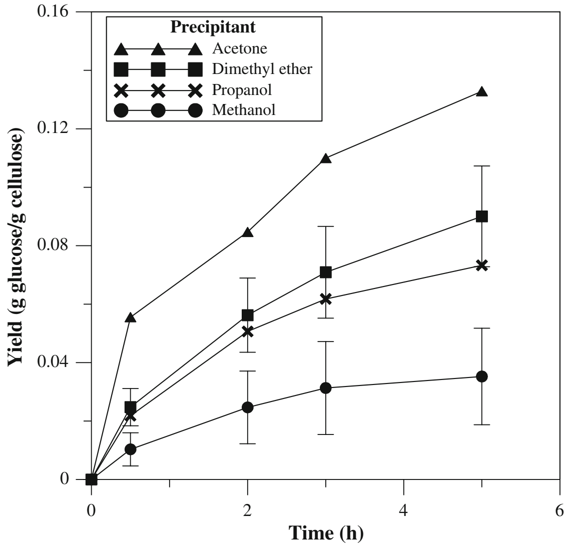

Fig. 1 Effect of type of precipitant on average yield at pH 5.0 and 45°C. Cellulose concentration 3% (w/v); glutaraldehyde at 10 mM. Not all error bars shown to avoid clutter

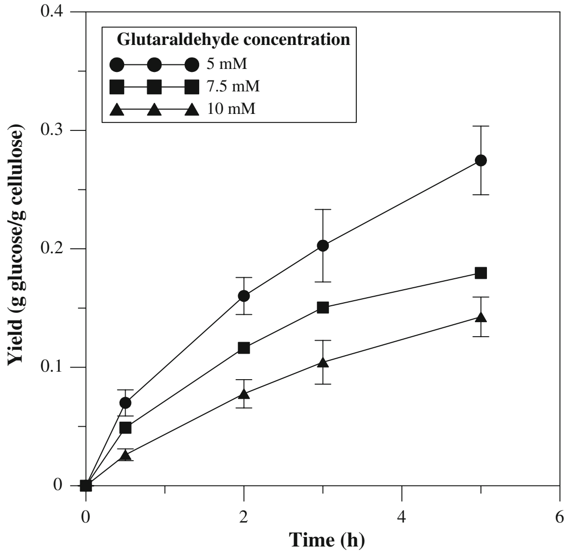

Fig. 2 Effect of glutaraldehyde concentration on average yield at pH 5.0 and 45°C. Cellulose concentration 3% (w/v)

cellulose after 5 h. Good cross-linking was achieved with 5 mM glutaraldehyde (Fig. 2); higher concentrations gave lower yields which was expected since glutaraldehyde inactivates cellulase.

The immobilized enzyme was reused five times (see Fig. 3). After an initial yield of 0.7 g glucose/g cellulose, the yield remained constant at 0.4 g glucose/g cellulose for the next three consecutive runs before decreasing to 0.3 g glucose/g cellulose in the fifth run.

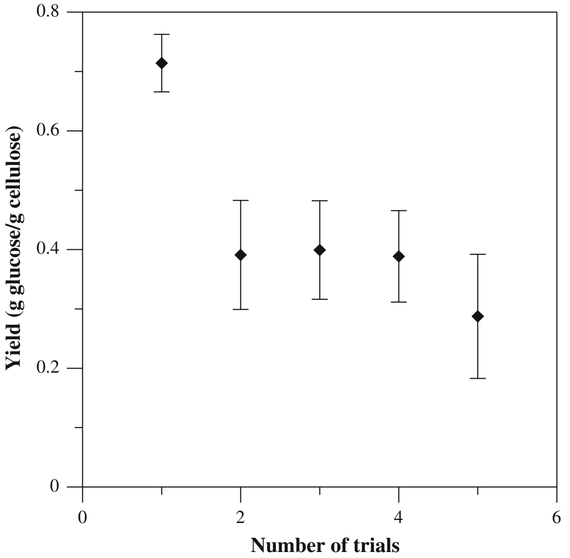

Fig. 3 Effect of reuse of immobilized enzyme on average yield of glucose at 24 h, pH 5.0 and 45°C. CFIC was prepared with 5 mM glutaraldehyde and acetone. Cellulose concentration 0.5% (w/v)

Ionic liquid pretreatment

EMIM-DEP is a low viscosity ionic liquid that has been successful in cellulose dissolution (Li et al. 2009). The initial reaction rate in the hydrolysis of crystalline cellulose was calculated over 2 h. Two% (v/v) EMIM-DEP gave the highest initial reaction rate (Table 1) which was 2.7 times higher than with no ionic liquid. With regenerated cellulose, Dadi et al. (2006) reported an initial rate enhancement of 2.8 times, based on glucose liberated within the first 3 h of reaction. With 20% (v/v) ionic liquid in the

Table 1 Effect of ionic liquid concentration on initial reaction rate of immobilized enzymatic hydrolysis of cellulose

EMIM-DEP used (% v/v)	Initial reaction rate (mg ml^{-1} min^{-1})	Enhancement factor
0	0.011	–
1	0.026	2.4
2	0.029	2.7
4	0.022	2.1

Glucose concentration was measured after 2 h at pH 5.0 and 45°C with initial cellulose concentration of 0.5% (w/v)

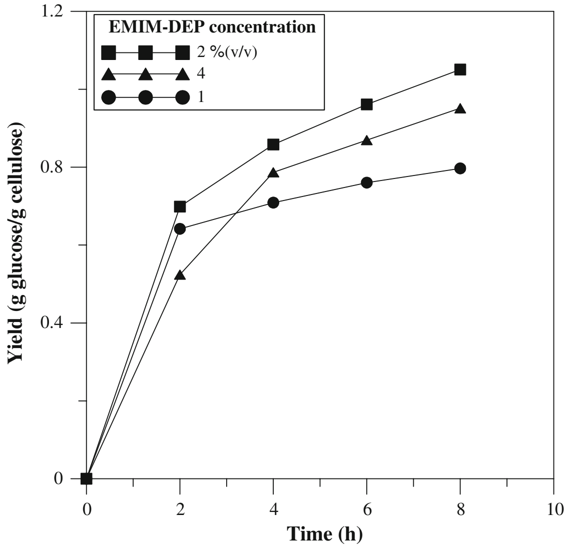

Fig. 4 Effect of concentration of ionic liquid (EMIM-DEP) in reaction media on average glucose yield at pH 5.0 and 45°C. CFIC was prepared with 5 mM glutaraldehyde and acetone. Cellulose concentration 0.5% (w/v)

reaction medium and with soluble enzyme, Kamiya et al. (2008) reported a nearly two-fold increase in cellulose conversion. In our work, we did not observe an increase in initial reaction rate above 2% (v/v) EMIM-DEP; on the contrary, the reaction rate decreased. However, after 8 h, the yields achieved with 2 and 4% (v/v) EMIM-DEP were close: 1.05 and 0.95 g glucose/g cellulose, respectively, as shown in Fig. 4.

Conclusions

Hydrolysis of crystalline cellulose by carrier-free immobilized cellulase in the presence of 2% (v/v) 1-ethyl-3-methylimidazolium diethyl phosphate resulted in an initial reaction rate that was 2.7 times the rate obtained with no ionic liquid. The carrier-free method employed in this study allows the immobilized enzyme to be effectively used five times.

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