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Effects of Ammonia Fiber Explosion Treatment on Activity of Endoglucanase from *Acidothermus cellulolyticus* in Transgenic Plant

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Abstract

A critical parameter affecting the economic feasibility of lignocellulosic bioconversion is the production of inexpensive and highly active cellulase enzymes in bulk quantity. A promising approach to reduce enzyme costs is to genetically transform plants with the genes of these enzymes, thereby producing the desired cellulases in the plants themselves. Extraction and recovery of active proteins or release of active cellulase from the plants during bioconversion could have a significant positive impact on overall lignocellulose conversion economics. The effects of ammonia fiber explosion (AFEX) pretreatment variables (treatment temperature, moisture content, and ammonia loading) on the activity of plant-produced heterologous cellulase enzyme were individually investigated via heat treatment or ammonia treatment. Finally, we studied the effects of all these variables in concert through the AFEX process. The plant materials included transgenic tobacco plants expressing E1 (endoglucanase from *Acidothermus cellulolyticus*). The E1 activity was measured in untreated and AFEX-treated tobacco leaves to investigate the effects of the treatment on the activity of this enzyme. The maximum observed activity retention in AFEX-treated transgenic tobacco samples compared with untreated samples was approx 35% (at 60°C, 0.5:1 ammonia loading, and 40% moisture). Based on these findings, it is our opinion that AFEX pretreatment is not a suitable option for releasing cellulase enzyme from transgenic plants.

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Index Entries: Ammonia fiber explosion; endoglucanase; *Acidothermus cellulolyticus*; transgenic tobacco; E1cd.

Introduction

In ethanol production, enzymatic hydrolysis of cellulose to glucose is an attractive route, because nearly theoretical yields of glucose are possible *(1,2)*. Cellulase enzymes are currently produced from microorganisms by expensive large-scale fermentation. The cost of enzyme-based processes has been reduced by about a factor of four *(1)*, and additional improvement opportunities have been identified that may make the technology even better *(3)*. However, enzyme production costs through microbial systems are still very high and tend to dominate the economics of enzyme-based bioconversion processes. These costs might conceivably be reduced by genetically transforming plants with cellulase genes to produce the desired enzymes, and perhaps even to release active cellulases from the plants during bioconversion.

Transgenic plants are an attractive and cost-effective alternative to microbial systems for production of biomolecules *(4)*. Advances in biotechnology are enabling plants to be exploited as bioreactors for the production of proteins, carbohydrates *(5,6)*, lipids *(7,8)*, and industrial enzymes *(9–11)* in bulk quantities with minimal inputs of raw materials and energy. As this technology continues to grow and improves the production efficiencies of biomolecules in plants, the development of downstream processing technology to extract and recover these biochemicals will increasingly determine progress in this area.Enzymes and other proteins produced in transgenic crops have very high value. Recovering and utilizing valuable bioactive plant proteins and enzymes in an overall process for producing fuels and chemicals from biomass may improve the lignocellulose conversion economics.To process cellulase-containing transgenic plants, the following technical options can be envisioned:

1. Harvest the cellulase-containing transgenic plants while they are green, macerate them, and separate the solid to produce an enzyme concentrate, which can later be used in the enzymatic hydrolysis of pretreated biomass. Depending on the production level of this enzyme in transgenic plants, the need for externally added cellulases in the enzymatic hydrolysis step might be avoided or minimized.
2. Harvest the cellulase-containing transgenic plants at the end of the season (dry), grind this material to release enzyme, and subsequently use it in the enzymatic hydrolysis of lignocellulosic biomass for ethanol production.
3. Harvest the cellulase-containing transgenic plants at the end of the season, use biomass pretreatment to rupture plant cells to facilitate the release of enzyme, and then combine this material with lignocellulosic biomass in enzymatic hydrolysis.

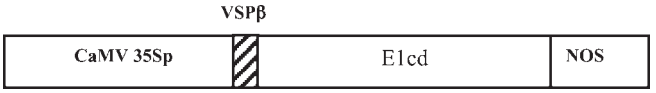

Fig. 1. Schematic representation of E1cd expression cassette. CaMV 35Sp, cauliflower mosaic virus promoter; NOS, nopaline synthase transcription termination signal; E1cd, catalytic domain of E1 gene; VSPβ, soybean vegetative storage protein β leader sequence to target the protein to apoplast *(15)*.

The success of any of these approaches depends on having a sufficiently high level of cellulase in the transgenic plant to make hydrolysis effective. Research such as that reported here is required to define which option(s) might prove favorable under specific circumstances.

A high recovery yield of plant proteins from biomass depends on extensive cell maceration. The more cell walls are disrupted, the more protein can be recovered *(12)*. Therefore, a pretreatment that disrupts plant cells may be useful in protein recovery processes.

An integrated pretreatment that improves protein recovery and increases the conversion of cellulose and hemicellulose to fermentable sugars could significantly enhance the biomass process economics. Many pretreatment processes that increase the conversion of cellulose to fermentable sugars operate under harsh conditions that tend to degrade the sugars, biomass proteins, and enzymes. However, previous studies *(13,14)* have shown that under limited treatment conditions, the AFEX process not only increases the conversion of cellulose and hemicellulose to simple sugars, but also allows the recovery of plant proteins in their native functional form.In the present study, we explored the third option that we just discussed. We investigated the potential of using AFEX as an integrated pretreatment process to enhance the release of active cellulase enzyme from transgenic plant while simultaneously increasing the digestibility of the biomass.

Material and Methods

Plant Material

Seeds of the transgenic tobacco plants expressing E1cd (catalytic domain fragment of E1 endo-1,4-β-glucanase from *Acidothermus cellulolyticus*) were obtained from Dr. Sandra Austin-Phillips from the University of Wisconsin–Madison. Figure 1 shows the plasmid that has been used by Dr. Austin-Phillips's group to transform tobacco plants to express the E1cd in apoplast.

Transgenic plants were grown, harvested (leaves), and dried at Michigan State University greenhouses. As Fig. 2 shows, the expression of E1cd did not cause any obvious phenotypic effect in plants and did not affect their normal growth.

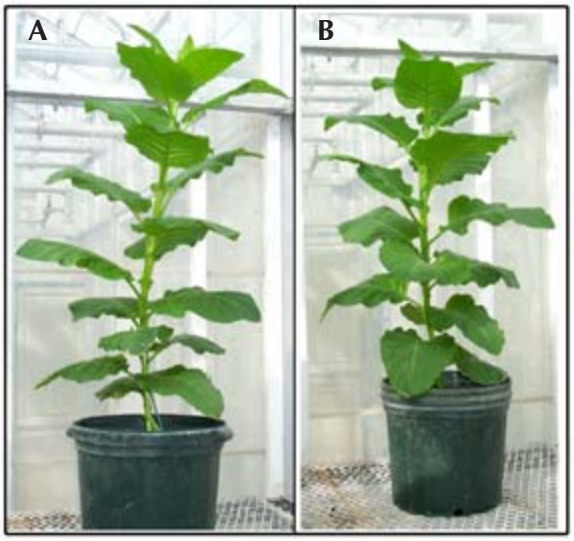

Fig. 2. **(A)** Nontransgenic and **(B)** transgenic tobacco plants grown in a greenhouse.

Identification of Transgenic Plant Expressing E1cd

After seedlings developed their first true leaves, samples were removed for enzyme assay to identify transgenic plants. Enzyme assay was conducted according to Ziegelhoffer et al. *(15)*. E1cd activity was determined by subtracting the background contributed by W38 control extracts, and fluorescence values were compared with values obtained with purified E1cd (provided by Steven R. Thomas, National Renewable Energy Laboratory [NREL], Golden, CO). The expression level of E1cd in transgenic plants was up to 2.5% of total soluble proteins. Transgenic plants were grown to maturity, and leaves were harvested, dried, and collected for AFEX treatment. Since each independently transformed plant had a different level of expression, the dried leaves were ground and well mixed before AFEX treatment to ensure the homogeneity of the samples.

AFEX Treatment

The AFEX reactor consisted of a 300 ml stainless steel pressure vessel (Parr Instrument, Moline, IL) (Fig. 3). The vessel was loaded with prewetted transgenic tobacco leaves with the desired moisture content (a range of moisture content was tested to determine the effect of moisture content on the activity of cellulase in AFEX-treated transgenic plants). The vessel was topped up with stainless steel pellets (approx 1 mm in diameter) to occupy the void space and thus minimize transformation of the ammonia from liquid to gas during loading, and then the lid was bolted shut. Using precalibrated ammonia sample cylinders, a predetermined amount of liquid ammonia was delivered to the vessel. The vessel was heated by a 400-W Parr heating mantle to the desired temperature.

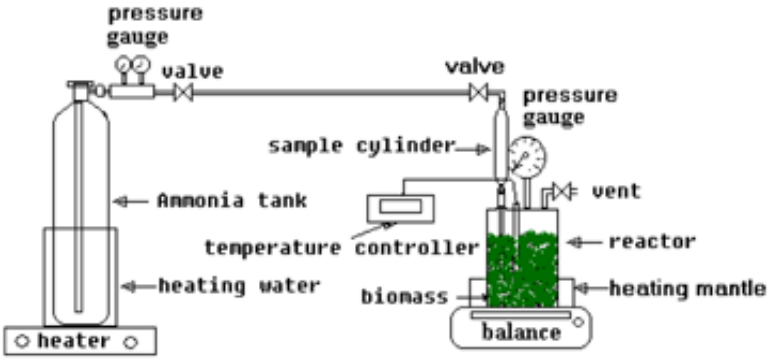

Fig. 3. Schematic diagram of laboratory AFEX apparatus.

After holding the vessel at the target temperature for 5 min, the exhaust valve was rapidly opened to relieve the pressure and accomplish the explosion. The treated samples were removed and left in a fume hood overnight at atmospheric conditions to evaporate the residual ammonia. AFEX-treated samples were darker than untreated sample, and other than this no visible physical change was observed.

Heat Treatment of Transgenic Tobacco Plants Expressing E1cd

The prewetted samples were placed in the AFEX unit, and the vessel was sealed and warmed to the desired temperature as described in the previous section. To avoid overheating, the reactor was taken out of the heater at approx 10°C less than the target temperature, and if needed the unit was placed in a bath of cold water to maintain the system at the set temperature. The system was kept at the target temperature for 5 min. Since there was no ammonia in this system, no increase in pressure was observed during the experiment. At the end of 5 min, the heat-treated samples were removed from the vessel and kept in plastic bags at 4°C until further analysis.

Ammonia Treatment of Transgenic Tobacco Plants Expressing E1cd

The prewetted samples were placed in the pressure vessel of the AFEX unit. The vessel was sealed as already explained. A predetermined amount of ammonia was delivered to the pressure vessel. To ensure that the desired amount of ammonia was delivered, the reactor vessel was weighed before and after loading. The vessel was left in the fume hood at room temperature (without heating) for 10 min. During the experiment, some of the liquid ammonia converted to gaseous ammonia and increased the pressure from 90 to 105 psi (after 10 min), and since the vessel was not heated, the system temperature was decreased (initial temperature was 34°C and final temperature was about 28°C).

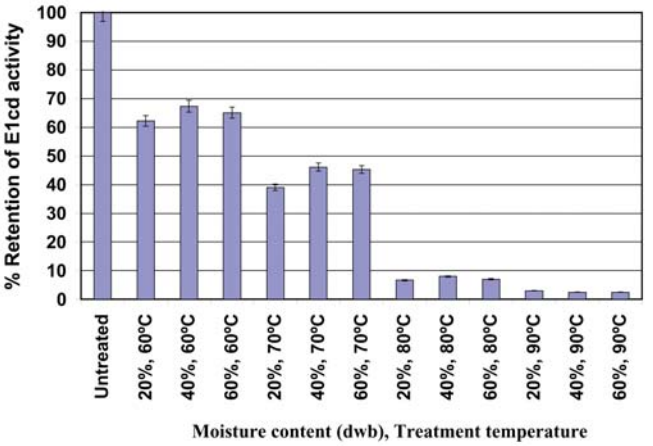

Fig. 4. Activity retention of E1cd extracted from heat-treated transgenic tobacco plants.

Measurement of Activity of E1cd Enzyme In Transgenic Tobacco Plants After Treatments

All the treated and untreated transgenic tobacco plant samples were ground and sieved to 40 mesh prior to the E1cd activity assay. E1cd was extracted from the treated and untreated samples as described by Ziegelhoffer et al. *(15)*. The total protein of each of the extracts was measured by the Bradford *(16)* method using bovine serum albumin as a standard. Subsequently the appropriate amount of extract containing the same amount of total protein was subjected to the activity assay.

Results and Discussion

Transgenic tobacco plants were treated at different temperatures (60–90°C) and at different levels of ammonia (0.5:1, 0.7:1, and 1:1 kg of ammonia:kg of dry tobacco sample) to assess the individual effect of each AFEX variable on the E1cd enzymatic activity. All results are the mean of two replicates, and they have been compared with the untreated transgenic tobacco sample. In all the runs the moisture contents are based on the sample dry weight.

Ziegelhoffer et al. *(15)* tested the stability of the apoplast-targeted E1cd in transgenic tobacco plants. In their study, the apoplast-targeted E1cd enzyme extracted from tobacco plants, along with the purified microbial E1cd, was subjected to different temperatures (60–90°C) for 10 min. Both enzymes showed similar high thermal stability throughout the experiment. Their results showed that at 60°C up to 95%, at 70°C up to 90%, at 80°C up to 80%, and at 90°C up to 40% of the enzymes' activity was retained. However, as seen in Fig. 4, our heat stability test showed sur-

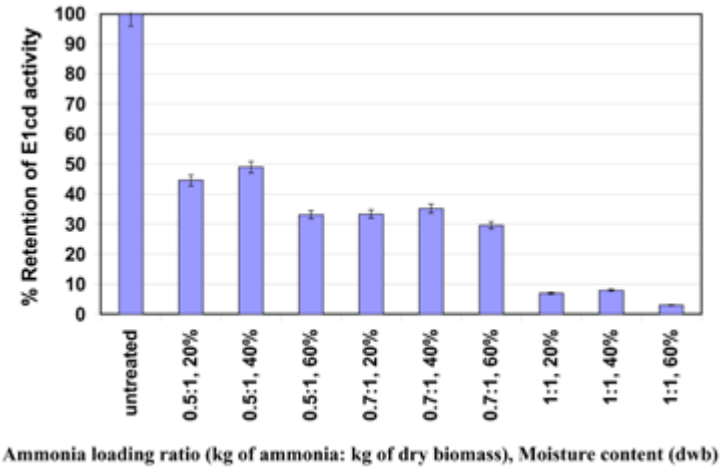

Fig. 5. Activity retention of E1cd extracted from ammonia-treated transgenic tobacco plants. dwb, dry weight basis.

prisingly different results from those reported by Ziegelhoffer et al. *(15)*. The E1cd extracted from transgenic tobacco plants treated at 60°C showed a maximum 67% and at 70°C showed a maximum 46% activity retention compared to E1cd extracted from untreated transgenic tobacco plants. The E1cd extracted from the samples treated at 80 and 90°C was almost completely inactive.

Figure 5 shows that at any moisture content, as the ammonia loading increased, the percentage of E1cd activity retention decreased, and at a 1:1 loading ratio, the E1cd enzyme was almost completely inactive. The highest percentage activity retention (49%) was observed at 0.5:1 ammonia loading with 40% moisture content. As Figs. 4 and 5 demonstrate, at any temperature and any ammonia loading, 40% moisture content showed the highest activity retention.

The transgenic tobacco samples were AFEX treated under different conditions. These experimental conditions were selected based on the results of our heat and ammonia treatment of transgenic tobacco plants; we chose the conditions that showed the highest enzymatic activity retention for E1cd. The results of these experiments are presented in Fig. 6.

E1cd enzyme showed better survival rates with individual heat or ammonia treatment (up to 67 and 49%, respectively), but the combination of heat and ammonia in AFEX treatment caused a drastic loss in the activity of E1cd. The maximum observed activity retention in AFEX-treated transgenic tobacco plants was only 35% at 60°C, 0.5:1 ammonia loading ratio, and 40% moisture content. Future studies may be able to clarify the mechanistic bases for these observations.

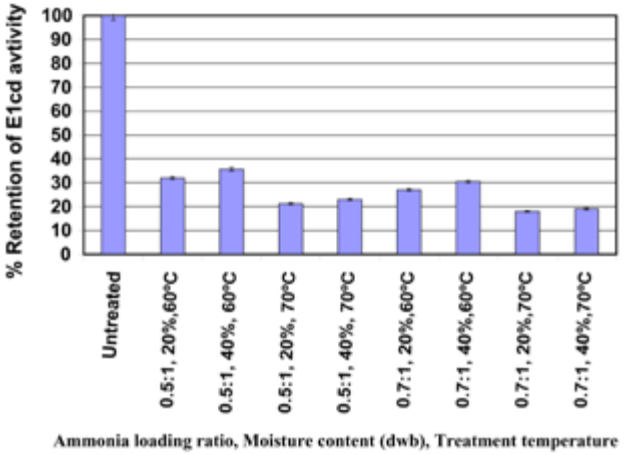

Fig. 6. Activity retention of E1cd extracted from AFEX-treated transgenic tobacco plants. dwb, dry weight basis.

Conclusion

Originally, pretreatment techniques were developed for various end uses of lignocellulosic biomass with an emphasis on ethanol production. Pretreatment processes have been used to enhance enzymatic hydrolysis of biomass to increase ethanol production and improve lignocellulosic conversion economics. Advances in biotechnology are enabling plants to become economically important systems for producing heterologous proteins such as cellulase enzymes. Therefore, expanding the application of biomass pretreatment to the release and recovery of these proteins might significantly improve biorefinery economics. We examined the potential of the AFEX treatment to recover active cellulase. As the data show, the maximum E1cd activity retention in AFEX-treated transgenic tobacco plant (under 60°C, 0.5:1 ammonia loading, and 40% moisture conditions) was only 35%. The glucan conversion of corn stover treated under the same conditions was only 47% of theoretical with 60 filter paper units/g of glucan of cellulase enzyme *(14)*. Based on these findings, it is our opinion that AFEX pretreatment is not a suitable option for releasing cellulase enzyme from transgenic plants. Considering the fact that other biomass pretreatments (e.g., steam explosion, acid treatment) operate under even harsher conditions, it is reasonable to assume that none of these pretreatments are a suitable choice for this purpose. Using pretreatment to release the cellulase enzymes from transgenic plants was only one of our technical options, therefore exploring the potential of other options (mentioned in Introduction) merits future study.

Based on these results and previous research *(15)*, we believe that E1cd extracted from transgenic plants is thermostable if it is heated in the

extraction buffer (at the enzyme's preferred pH of 5.5), but its thermal stability drastically decreases if plants expressing the enzyme are heated directly.

Acknowledgments

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