A green solution for ethanol production

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The use of ethanol as an automotive fuel dates back to the early 20th century, with a memorable example being Henry Ford's famous T model that was able to run on pure ethanol. With increased interest in renewable resources, the use of bioethanol as an automotive fuel on a global scale has been increasing [1, 2]; however, the long-term viability of ethanol as a renewable fuel is challenged by the availability of suitable feedstocks that can be used for the fermentation process [1].

Currently sugar, molasses and grain starch, particularly starch from corn, are the major feedstocks used for large-scale ethanol production. If demand for fuel ethanol continues to grow and there remains support from governments in the form of subsidies and mandated renewable targets, ethanol production will need to shift to non-edible sources for feedstock in order to avoid the fuel-food competition [2]. In the long run, there is a need to develop cost-effective and competitive large scale production of ethanol from lignocellulosic feedstocks, which are the non-grain or non-tuber part of the crop plant. The use of residues from genetically modified crop plants may constitute an important feedstock source for the production of ethanol as a viable renewable automotive fuel. To that end, expression and accumulation of cellulose degrading enzymes in plant cells may provide a valuable tool in tackling this challenge.

Ethanol can be produced from lignocellulose feedstocks, but it currently relies on the addition of exogenous enzymes to digest the long cellulose strands into oligosaccharide fragments prior to microbial fermentation of these oligosaccharides to ethanol. Improving the efficiency of this process by optimising the thermal and chemical stability of the enzymes, and the developing technologies required to meet the enormous production capacities will be required if lignocellulosic ethanol is to become the major feedstock from renewable sources [3]. This efficiency presents microbiologists, biotechnologists, enzymologists, fermentation researchers and engineers with a number of challenges.

In the past decade there has been a significant push to improve the pre-digestion processes used for lignocellulose ethanol fermentation. Considerable funding, particularly in the US, both private and government subsidised, has been directed towards the discovery of novel microbial cellulose digesting enzymes and attempts to improve the enzymatic characteristics of those cellulose enzymes traditionally used in the pre-digestion steps. Approaches have included classical mutagenesis, sited-directed mutagenesis and, more recently, even forced evolution or 'gene shuffling' approaches have been applied to this problem [4].

There are reports of some success in modifying the kinetics of cellulase and improving the suitability of the enzymes for industrial application [5, 6], with much of this work driven by corporations involved in large scale enzyme production. However, even with the reported improvements in enzyme kinetics, the economic costs associated with the use of these enzymes in the production process still account for almost 30% of the overall cost of the production, making lignocellulose derived ethanol not economically viable. Furthermore, the capacity of the enzyme production companies to scale up enough fermentation capacity in order to supply the quantities of enzyme needed to produce the billions of litres of ethanol as a reliable fuel alternative is not currently sufficient. In this regard, the possibility of producing an E85 (an 85% ethanol blend) type fuel source on a global scale is unlikely, if not impossible.

An alternative approach that has been explored is the use of transgenic plants that express the genes encoding cellulolytic enzymes [7]. The proposal is that the transgenic plant derived biomass would contain at least part of the enzyme cocktail normally added to the plant biomass, thereby eliminating the need to add the enzymes exogenously during the ethanol production process. The key to this proposal is the expression and accumulation of the appropriate combination of cellulase enzymes in the transgenic plant. The *'in planta'* expression of cellulase genes has been reported in a number of different

plant species, including maize, alfalfa and rice [7-9]. However, several significant challenges still exist in taking these laboratory achievements to systems that could be possibly considered as the basis for an industrial process.

First there needs to be expression of the right combination of exocellulase, endocellulase, hemicellulase and possibly lignin degrading enzymes that are required for the predigestion of the plant biomass. A second challenge is to ensure that the expression and accumulation of these enzymes does not damage or alter the host plant during plant development, particularly if crop plants are to be targeted as sources of biomass. Third, and perhaps most challenging, is the expression of the cellulase enzymes at levels high enough and in the appropriate ratio to make auto-digestion of the cellulose feedstock technically feasible and economically viable.

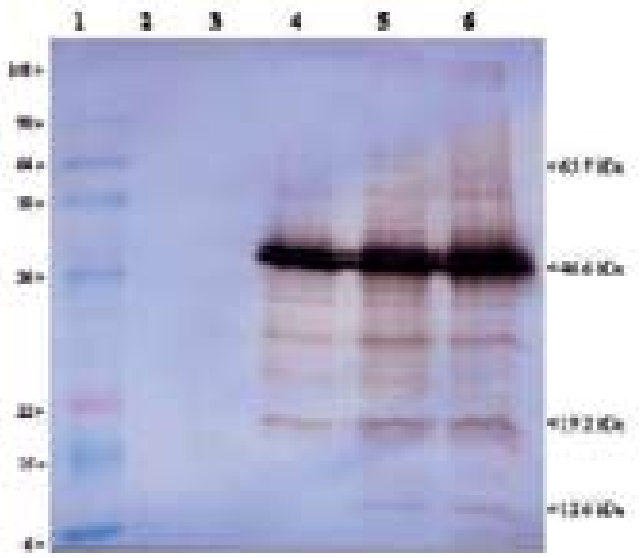

Figure 1. Western Blot analysis of proteins from transgenic plants expressing E1 cellulase genes.

Western Blot analysis of protein extracts from the transgenic plants was carried out with monoclonal antisera raised in mouse against *A. cellulolyticus* E1. A reactive band is clearly visible at 46.6kDa in all three samples. This band corresponds to the catalytic domain of the E1 enzyme. Two other reactive bands are visible in the samples taken from the dual transgenic plants, one at 65.7kDa and another at 12.6kDa. These correspond to the expected sizes of full-length E1 enzyme and the cellulose binding domain of E1 respectively.

Lane 1 – Protein Molecular Weight Marker SeeBlue® Plus2 Pre-Stained Standard (Invitrogen)

Lane 2 – empty

Lane 3 – wild-type *N. tabacum* protein extract

Lane 4 – nuclear transformed and apoplast targeted E1 transgenic plant extract

Lanes 5 & 6 – dual nuclear and apoplast targeted E1 and chloroplast transformed E1 transgenics plant protein extracts.

Transgenic plant expression, with sub-cellular targeting, is an approach that has been successfully used to increase levels of transgene expression in plants, with high levels of cellulase enzymes having been achieved with apoplast targeted cellulase [8]. Another sub-cellular target that has been examined is the plant chloroplast, and transfer and expression of the cellulase genes within the plant chloroplast itself has also been examined [10].

Our recent results, using the *Acidothermus cellulolyticus* endoglucanase E1 gene, have shown that it is possible to combine nuclear transformation with the cellulase enzyme directed to the apoplast for accumulation, and chloroplast expression and retention, within the same plant (Figure 1). This combined expression approach can result in E1 cellulase protein levels of approximately >5% of total soluble protein, with the enzymes retaining enzymatic activity. Achieving this sort of targeting and accumulation of cellulase protein is an important step towards applying *in planta* expression of cellulase enzymes to the problem of industrial scale enzyme-mediated predigestion of lignocellulosic feedstocks. A major challenge for the future will be the extension of this technology to some of the broad acre crops that currently produce the millions of tonnes of biomass residue on a yearly basis. These broad acre crops currently constitute the only renewable source of lignocellusic feedstock on the planet that can sustain a fuel ethanol industry on a sustainable and global scale.

References

1. Service, R.F. (2007) Cellulosic ethanol. Biofuel researchers prepare to reap a new harvest. *Science* 315, 1488-1491.
2. Lenk, F. *et al.* (2007) On the usage of agricultural raw materials: energy or food? an assessment from an economics perspective. *Biotechnol. J.* 2, 1497-1504.
3. Mielenz, J.R. (2001) Ethanol production from biomass: technology and commercialization status. *Curr. Opin. Microbiol.* 4, 324-329.
4. Tao, H. and Cornish, V.W. (2002) Milestones in directed enzyme evolution. *Curr. Opin. Chem. Biol.* 6, 858-864.
5. Percival Zhang, Y.H. *et al.* (2006) Outlook for cellulase improvement: screening and selection strategies. *Biotechnol. Adv.* 24, 452-481.
6. Rosgaard, L. *et al.* (2006) Efficiency of new fungal cellulase systems in boosting enzymatic degradation of barley straw lignocellulose. *Biotechnol. Prog.* 22, 493-498.
7. Yu, L.X. *et al.* (2007) Expression of thermostable microbial cellulases in the chloroplasts of nicotine-free tobacco. *J. Biotechnol.* 131, 362-369.
8. Hood, E.E. *et al.* (2007) Subcellular targeting is a key condition for high-level accumulation of cellulase protein in transgenic maize seed. *Plant Biotechnol. J.* 5, 709-719.
9. Oraby, H. *et al.* (2007) Enhanced conversion of plant biomass into glucose using transgenic rice-produced endoglucanase for cellulosic ethanol. *Transgenic. Res.* 16, 739-749.
10. Streatfield, S.J. (2007) Approaches to achieve high-level heterologous protein production in plants. *Plant Biotechnol. J.* 5, 2-15.

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