

## BIOTECHNOLOGICAL TOOLS TO IMPROVE ETHANOL PRODUCTION FROM PLANT BIOMASS

JURAJ FARAGO<sup>1,2</sup>

<sup>1</sup>*Slovak Agricultural Research Center, Research Institute of Plant Production,  
Bratislavská cesta 122, Piešťany, SK-921 68, Slovak Republic (farago@vurv.sk)*

<sup>2</sup>*Department of Biotechnology, University of SS. Cyril and Methodius, J. Herdu 2,  
Trnava, SK-917 01, Slovak Republic*

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**Abstract:** Increasing concerns for security of the fossil fuel supply emphasizes the need to complement fossil fuel-based energy sources with renewable energy sources. Plant biomass represents an abundant renewable resource for the production of bioenergy and biomaterials. This review summarizes the last advancements in the use of biotechnological tools to improve bioethanol production from plant biomass through genetic engineering the starch content and composition and lignocellulosic matter characteristics, and increasing the capacity of plants to produce harvestable yield and ameliorating the negative abiotic stresses on plants so as to increase yield.

**Key words:** biotechnology, genetic engineering, starch, lignocellulose, crop yield

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### 1. Introduction

The production of ethanol for use as a transportation fuel is not a new technology. It was first introduced in the U.S.A. in the 1930s, when the Ford Model T was able to run on either gasoline or ethanol (KOVARIK, 1998). Post World War II, however, the interest in the use of agricultural crops for ethanol production dropped, mainly because of the availability of cheap and abundant fuel from fossil sources. Renewed interest in ethanol as fuel developed in the 1970s (BOTHAST and SCHLICHER, 2005). Brazil was the world's first country to run large-scale program for using ethanol as fuel (DE OLIVEIRA *et al.*, 2005), and in the United States ethanol has been utilized as a fuel source since the turn of the century (BOTHAST and SCHLICHER, 2005). In Slovak Republic, the production of corn-based bioethanol started in this year (P. KOSTÍK, Enviral, personal communication).

It is now understood, that it is important to use plant biomass energy, that would complement solar, wind, and other intermittent energy sources. Biomass is considered an interesting energy source for several reasons (see LIN and TANAKA, 2006). Most agricultural biomass containing starch or sucrose can be used as a substrate for ethanol fermentation by microbial processes. These "energy crops" include maize (corn), wheat, rice, potato, cassava, sugarcane and sugarbeet.

Currently used crops, however, are non-optimized for biofuel production. Thousands of years of traditional breeding through phenotypic selection have resulted in modifications that are better fit for harvesting for feed or food uses (McLAREN, 2005).

Genetic engineering can offer viable opportunities to increase bioethanol production from crop plants principally in two ways: modifying the biomass properties

and/or increasing biomass yield. This article will review the latest advancements in the research and development focused on these two topics.

## 2. Biomass properties

### 2.1 Starch content and structure

Starch is by far the most important storage carbohydrate found in plants. Annually, 20-30.10<sup>6</sup> tons of starch are produced to serve a wide range of industrial applications such as coating of textiles and paper, or as a thickening or gelling agent in the food industry (HEYER, 2000). Most of the starch is isolated from corn, but also potato, wheat and cassava are sources of starch.

Starch is deposited in the plastidic compartment of plants as granular material, for example in the amyloplasts of plant storage organs, such as seed endosperms or tubers. It is composed of two glucose polymers, amylose and amylopectin. In the linear polymer amylose, the glucose monomers are linked by  $\alpha$ -1,4-glycosidic linkages. In contrast, the molecule of amylopectin is branched and various amount (about 5% in maize) of its glucose units are linked by  $\alpha$ -1,6-glycosidic linkages. The molecular weight of amylopectin is higher (10<sup>7</sup>-10<sup>8</sup> Da) than that of amylose (10<sup>5</sup>-10<sup>6</sup> Da). Normally, starches contain 70-80% amylopectin in contrast to 20-30% amylose (HEYER *et al.*, 1999).

The biosynthesis of starch is sufficiently understood to allow genetic engineering of plants for higher accumulation or modified composition. Alteration of starch structure and content can be achieved by modifying genes coding for enzymes responsible for starch synthesis (HEYER, 2000; JOBLING, 2004). Transgenic plants with modified expression of different starch synthases, starch branching enzymes or starch debranching enzymes have been produced to increase the accumulation of starch (TJADEN *et al.*, 1998; MCKIBBIN *et al.*, 2006) or to alter the crystallinity of starches and thus increase the accessibility to enzymatic digestion (BAGA *et al.*, 1999; OTANI *et al.*, 2007). STARK *et al.* (1992) demonstrated an increase of up to 135% of control starch content was obtained following the constitutive expression of a regulatory variant of bacterial ADPglucose pyrophosphorylase (AGPase). Another possibility is to decrease the gelatinization temperature of modified starches to reduce the energy requirement for the conversion of starch to ethanol. Recently, for example, CHIANG *et al.* (2005) showed that overexpression of a thermostable and bifunctional starch hydrolase, amylopullulanase (APU) from *Thermoanaerobacter ethanolicus* 39E in rice seeds resulted in starch, that could be hydrolyzed with optimal temperatures between 85 and 95 °C to complete conversion into soluble sugars.

### 2.2 Lignocellulosic biomass

Lignocellulosic biomass is renewable, cheap and readily available with over over 10-50.10<sup>9</sup> tons produced per year at the global level (STICKLEN, 2006). Lignocellulose is a more complex substrate than starch. It is composed of a mixture of different carbohydrate polymers (cellulose and hemicellulose) and lignin.

The biological process for converting the lignocellulose requires several steps: (1) delignification to liberate cellulose and hemicellulose from their complex with lignin, (2) depolymerization of the carbohydrate polymers to produce free sugars, and (3) fermentation of mixed hexose and pentose sugar to produce ethanol (LIN and TANAKA, 2006). The major costs of biomass refineries include the pre-treatment processing of the lignocellulosic matter at the delignification step, and the cost of production of the microbial cellulases needed to convert the cellulose biomass into fermentable sugars (KABEL *et al.*, 2005; STICKLEN, 2006).

Genetic engineering provides tools to decrease both of these costs. Several approaches have been used to accomplish these goals. Firstly, genetic transformation of plants can decrease the lignin content and/or change the composition of lignin. Lignins are complex three-dimensional polymers embedded in the cell walls of plant cells. In order for cellulases to access the cellulose for degradation, costly and harsh heat or acid pretreatment of biomass is required to remove lignin and hemicellulose from the lignocellulosic matter. Transgenic manipulation of different lignin biosynthetic pathway genes have been attempted to decrease lignin content (PIQUEMAL *et al.*, 2002; CHEN and DIXON, 2007) or alter lignin composition (RALPH *et al.*, 2006) and thus reduce the pre-treatment costs. Secondly, transgenic plants can be designed to overexpress enzymes for cellulases, such as endoglucanases, exoglucanases and  $\beta$ -glucosidases, or produce recombinant (microbial) cellulases within the biomass crop. Recently, MONTALVO-RODRIGUEZ *et al.* (2000) have used this approach to generate transgenic plants constitutively producing either a hyperthermophilic  $\alpha$ -glucosidase or  $\beta$ -glucosidase using genes derived from the archeon *Sulfolobus solfataricus*. The authors showed that transgenic plant protein extracts released glucose from purified polysaccharide substrates at appreciable rates during incubation in high-temperature reactions. In another study, ORABY *et al.* (2007) constitutively expressed the catalytic domain of *Acidothermus cellulolyticus* thermostable endoglucanase gene (E1) in rice and observed that approximately 30% of the cellulose of Ammonia Fiber Explosion-pretreated maize biomass was converted into glucose using rice E1 heterologous enzyme. Lastly, genetic engineering can be employed to produce recombinant microbial cellulases and/or ligninases in bioreactors (e.g. GALLIANO *et al.*, 1988, JIN *et al.*, 2004).

### 3. Biomass yield

Biomass yield is a very complex trait. Unlike the genes for starch, cellulose and lignin biosynthesis, the majority of genes involved in the yield traits remain elusive. Therefore, it was believed, that yield as a trait can not be easily manipulated through genetic engineering approaches. However, current achievements in genetic transformation technology suggest, that biomass yield increase and stabilization can be achieved through understanding and enhancing such mechanisms as stress tolerance (VINOCUR and ALTMAN, 2005, UMEZAWA *et al.*, 2006), nitrogen assimilation (HARRISON *et al.*, 2000), and carbohydrate metabolism (SAKAMOTO and MATSUOKA, 2004). Crop yields can be increased by increasing leaf photosynthetic rates. Considerable effort has been focused, for example, on molecular modification of

photosynthesis by introducing the precursor pathway for organic acid fixation of CO<sub>2</sub> (C4 pathway) into C3 species (MATSUOKA *et al.*, 2000; EVANS and VON CAEMMERER, 2000). Another way to improve photosynthesis may be modifying plant architecture to adjust to the high planting density currently used in agriculture (SAKAMOTO and MATSUOKA, 2004). In cereals, grain yield is the main target for genetic improvement. ADPglucose pyrophosphorylase is frequently referred as the rate limiting enzyme in starch biosynthesis and as such the key enzyme regulating sink strength. Recent reports on transgenic deregulation of AGP suggest a possibility to increase plant sink strength and subsequently the seed and biomass yield (SMIDANSKY *et al.*, 2003, 2007).

Enhanced stress tolerance in plants through genetic engineering has been achieved through manipulation of effector genes (e.g. biosynthetic enzymes, ion transporters) or regulatory genes (e.g. transcription factors or signal transduction components) (FAO, 2001).

#### 4. Conclusion

In the future, fuel ethanol will remain an attractive option, at least because it is a renewable energy source, creating a substantial new market for different high energy crop supplies and new jobs. Genetic engineering can effectively contribute to and provide great potential for future agriculture and biofuel production. However, to fully exploit the potential of modern biotechnology tools, it is necessary to increase the public acceptance of biotech-derived crops. Also, in many countries the legislation concerning plant biotechnology and the regulatory system lags behind the advancement of a technology. However, genetic engineering to improve biomass characterization will be economically feasible if the social benefits from the adoption of the technology will be greatest than its social costs.

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