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### ABSTRACT

The first stage in the production of cellulosic biofuels is the release of sugars from the biomass. Sugar beet pulp (SBP) offers a potential source of sugars provided they can be released within the parameters of the sucrose production campaign. For SBP it will be advantageous to utilize the diffuser processing temperature to speed up the enzymatic degradation. We have engineered thermostabilizing mutations into the *Pectobacterium chrysanthemi* pectin methylesterase (PME). A version (JL25) with four amino acid substitutions raised the stability over the wild-type by close to 12 C to 62 C. In addition to contributing to temperature stability, three of the four mutations resulted in minor improvements in the enzyme's catalytic constant (kcat). The initial velocities catalyzed by the wild-type and thermostabilized enzymes are comparable at the temperature optimum (50 C) of the wild-type enzyme. However, at 60 C the initial velocities are approximately 140% of those of the wild-type at its optimum temperature. Pectinex® Ultra SPL (Novozymes) is a mixture of enzymes derived from *Aspergillus aculeatus*.

Sugar beet pulp, untreated except for chopping in a Waring blender, was digested with a widely used commercial pectinase preparation, Pectinex ULTRA SPL with and without added JL25 PME. Briefly, 50 g wet weight of chopped pulp was mixed with 26,000 units Pectinex ULTRA SPL or 26,000 units Pectinex ULTRA SPL with 0.5 ug of PME, in 100 mL of 50 mM MOPS buffer pH 6.5. The samples were digested at 55° C (the upper temperature limit of the pectate lyase in the Pectinex) in a rotating cylinder for 48 hr. Following digestion the mixture was pressed into a water soluble fraction and a solid residue. The soluble fraction was assayed for total reducing sugars by the dinitrosalicylic acid (DNS) method and analyzed by HPLC for individual sugar concentrations. The residue was dried to remove water and weighed.

With the addition of our engineered PME to the Pectinex®, and without further chemical or heating pre-treatments, we solubilized approximately 90% of the theoretically available sugars from the arabinose and galacturonic acid contained in the beet pulp (~70% of total available sugars). Between the soluble sugars and the remaining pressed solid residue we accounted for approximately 90% of the total mass. It is likely the missing mass is due to the presence of soluble, oligomeric sugars that the DNS assay would underestimate. Further, at 55 C the initial rate of sugar production from beet pulp was approximately 50% greater in the PME-added vs Pectinex® alone.

Following these results the enzyme cocktail was augmented by the addition of a commercial cellulase preparation. Chopped sugar beet pulp was digested as described above with 26,000 U Pectinex, 0.5 ug JL25 PME with or without 40 Filter paper units of cellulase (NS50013, Celluclast – Novozymes). After 24 hr the digestion products were processed as described above and an accounting of the sugars and total mass was undertaken. Total, soluble reducing sugar release went from 55% (Pectinex and Celluclast) to 70% (Pectinex, Celluclast and JL25 PME) with between 89 – 94% of total

mass accounted for. After 48 hr digestion with Pectinex, Celluclast and JL25 PME 80% of theoretically available sugars were found in the soluble fraction.

In addition to these sugar hydrolysis results, varying the time periods and enzyme mixtures used to treat beet pulp identified glucose inhibition effects on hydrolysis of both pectin and arabinose. By changing the order of enzyme treatment, these inhibition effects were minimized leading to higher sugar solubilization rates for galacturonic acid, arabinose, and glucose.

The five carbon (C-5) sugars produced from the hemicelluloses biomass and the six carbon sugars (C-6) produced from the cellulose and pectin biomass can all be used in the production of biofuels. This includes fermentation for ethanol production and more advanced processes such as aqueous reforming that convert sugars to alkane hydrocarbons.

These results can be developed into a low capital biofuel precursor production sub-system that can be integrated into existing sugar beet processing facilities. Such a system would allow sugar beet processors to convert low-value wet pulp into high-density, medium value sugars that could be marketed to a variety of current and emerging advanced biofuel producers.