Improved Hydrolysis of Pretreated Lignocellulosic Biomass using Mobile Enzyme Sequestration Platforms

Ruben Michael Ceballos*, Ruben Michael Ceballos Jr., Asha Rani, Chelsea Teresa Morales, and Natalia Alekseyevna Batchenkova

***Abstract*—**On a large commercial scale, cellulosic ethanol, a "second-generation" biofuel, has yet to become cost competitive with fossil fuel-based liquid fuel products (e.g., gasoline). In an effort to lower the per-gallon production cost of bioethanol, two stages within the production process have been the subject of intense investigation: pretreatment; and, enzymatic hydrolysis. In general, the development of advanced pretreatments is aimed at disrupting interactions between cellulose, hemicellulose, and lignin as well as enhancing the exposure of the cellulose network to sugar reducing (e.g., hydrolytic) enzymes. The identification of novel enzymes, the engineering of enzymes, and the use of platforms that enhance enzymatic efficiency comprise a second area of research focus. In this study, we present data showing that the use of a NASA-developed mobile enzyme sequestration platform (mESP) is effective in enhancing sugar reduction efficiency on acid- and alkaline- pretreated cellulosic feedstock. Further advancement of this technology could contribute to the development of a cost-competitive cellulosic ethanol product.

***Keywords*—**bioethanol, cellulosic ethanol, enzyme platforms, lignocellulose degradation

I. Introduction

THE production of ethanol as a supplemental transportation fuel, and a partial substitute for gasoline, is ongoing. Feedstocks such as corn and sugarcane are the basis for more than 90% of the world's current fuel ethanol supply [1], [2]. The use of *first-generation* feedstocks, which provide sucrose and starch, has been the subject of much debate since the production of ethanol from these sources can impact food supplies, in what is called the *food-versus-fuel* dilemma [3].

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An alternative to corn, sugarcane, and other first-generation feedstocks are *second-generation* feedstocks, which include forms of lignocellulosic biomass such as corn stover, bagasse, crop straws, perennial energy crops (i.e., grasses), woody energy crops (e.g., cottonwoods, poplar, bamboo), and forestry waste. Second-generation lignocellulosic biomass is arguably the most promising type of feedstock for bioethanol production due to its global abundance and availability. However, the conversion of lignocellulosic secondary substrates (i.e., cellulose and hemicellulose) to primary sugar substrates (e.g., glucose) for fermentation processes is challenging from an economic standpoint. Although multiple technologies exist for reducing complex carbohydrates, such as cellulose, to simple fermentable sugars, such as glucose, the costs associated with these conversion processes have limited wide-scale commercial production of cellulosic ethanol.

Two steps within the ethanol production process that have been studied in an attempt to reduce the per-gallon production cost of bioethanol are the feedstock pretreatment step and the enzyme-mediated sugar reduction step. Recent reviews [4], [5] and research articles [6]-[10] have been published on the latest advancements in pretreatment technology. In general, pretreatment technology is aimed at: partially dissolving the lignocellulosic matrix; disrupting cellulose, hemicellulose, and lignin interactions; and, exposing the cellulose structure for subsequent to enzymatic hydrolysis using sugar reducing enzymes (e.g., cellulases), which can degrade complex large carbohydrates to simpler fermentable sugars (e.g., glucose).

The enzymatic step within the bioethanol production process has also been a research focus. Four approaches have been undertaken to improve sugar reduction efficiency. First, discovery of novel cellulases and other lignocellulose degrading enzymes through bioprospecting has led to the identification of enzymes [11] that function more efficiently under conditions that are used in most bioethanol production processes (e.g., elevated temperatures and in acidic solutions). Second, researchers have attempted to alter the reaction conditions and employ enzyme "cocktails" to optimize enzymatic activity [12], [13], [14] while maximizing flow-through rates and end-product yields. Third, scientists have genetically altered or otherwise designed cellulose deconstruction enzymes that have high catalytic efficiency [15], [16], [17] under production conditions.

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All of these approaches have met with some success; however, the level of enhancement has not led to significant reduction in production costs such that cellulosic ethanol is competitive with either first-generation ethanol or fossil-based liquid fuels. Although many of these enzyme technologies are viable from a technical perspective, the cost of producing such enzymes and the enzyme loads required for commercial scale production are economically prohibitive.

A fourth approach to enhance sugar reduction is to bolster enzymatic hydrolysis via the use of platforms [18], [19], [20]. Enzyme platform approaches stem from understanding the manner in which natural systems function. Specifically, cellulose degrading microorganisms, such as bacteria and fungi, employ expansive protein "scaffolds" to degrade cellulosic biomass. These large protein complexes are referred to as *cellulosomes* [21] and some researchers have attempted to mimic the natural cellulosome either wholly or in part to enhance sugar reduction efficiency in industrial processes. (Some research focused on enhancing hydrolysis using living microbes that express cellulosomes has also been conducted with limited success [22] - a fifth approach). These artificial cellulosomes have evolved into protein platforms that only vaguely resemble natural cellulosomes. Although, the same enzymes that are found in natural cellulosomes are often used in engineered enzyme platforms [19], as designer enzymes become more prevalent these artificial constructs less and less resemble natural cellulosome systems.

Indeed, platform technology can have several forms. Immobilized platforms bind lignocellulose deconstruction enzymes to columns or other surfaces that allow slurry to pass through or over an enzyme array [23], [18], [24]. Hydrolysis takes place as the, often pretreated biomass moves along the array and bound enzymes interact with substrate. This approach has been met with limited success [25], [26]. Although enzyme loading is more closely controlled using immobilized platforms, thereby reducing the loss of expensive enzymes to waste, accessibility – specifically, the ability of enzyme to thoroughly penetrate the biomass – is limited. This requires enhanced mixing strategies and slow perfusion rates.

More recently, the development of mobile platforms has emerged as a potential solution [19], [20]. One of the more promising constructs, a prototype called a "rosettazyme", was developed in 2007-2009 as a mobile platform that could bind sugar reducing enzymes and still move through slurry as a large protein complex. At the core of the platform is an altered double-nonameric ring (18-mer) heat shock protein complex derived from a hyperthermophilic archeaon of the genus *Sulfolobus* (*see* Figs. 1-3). The subunits of the complex were modified to bind cellulosome enzymes of the cellulose-degrading bacteria *Clostridium thermocellum*. This mobile enzyme sequestration platform (mESP) was tested on an over-the-counter cellulose product – Avicel® [19]; however, it was never tested on actual pretreated feedstock.

After the project had been terminated at NASA, our lab reconstituted the system and tested it on actual feedstock. In this study, we demonstrated enhanced sugar reduction efficiency on pretreated substrate using this mESP technology.

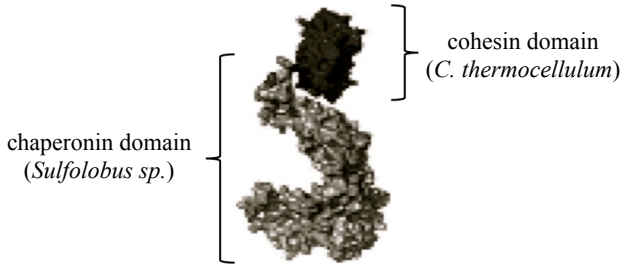

Figure 1 – Pymol model of HSP-coh fusion protein. Circular permutant of a group II chaperonin "heat-shock" protein (grey) from the archaeon Sulfolobus sp. (lab strain) linked to the cohesin (Type 1) protein (black) from the bacterium Clostridium thermocellum. (*Image adopted and modified from [19] with permission)*

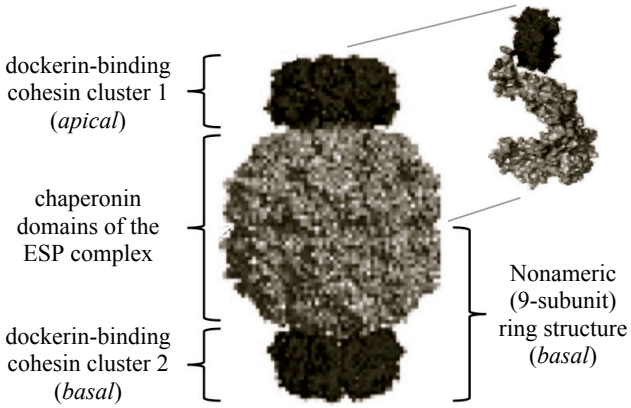

Figure 2 -Pymol model of ESP complex. Nine HSP-coh subunits comprise each of two ring structures that constitute the 18-mer ESP. Cohesin (type 1) domains from each subunit cluster at apical and basal extremeties and are capable of binding enzymes equipped with a dockerin (type 1) domain. This model illustrates the "uncharged" (no bound enzymes) configuration. (*Image adopted and modified from [19] with permission)*.

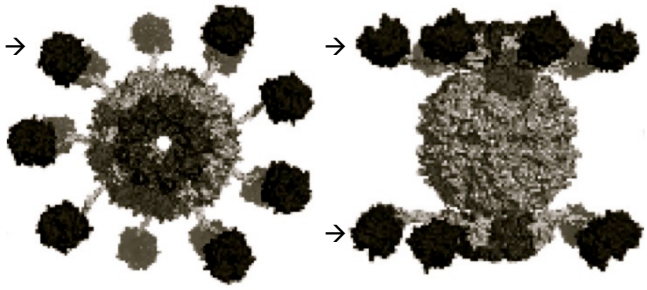

Figure 3 -Pymol model of enzyme-charged ESP complex. Top view (*left*) of an ESP complex with dockerin-containing enzymes bound. Side view (*right*) of an ESP complex showing enzyme binding (*arrows*) at apical and basal cohesin clusters. (*Image adopted and modified from [19] with permission).*

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II. Methods and Materials

A. mESP construction

As detailed in [19], a fusion protein was constructed using recombinant DNA methods. This fusion protein, HSPβ-coh (Fig. 1) is composed of a circular permutant of HSPβ [27], an archaeon (*Sulfolobus sp*.) heat-shock protein, and the cohesin module of CipA from the bacterium *Clostridium thermocellum* (residues 179-325; NCBI Q06851). This fusion construct contains a nine (9) amino acid linker (i.e., GGSGGSGGS) between the HSPβ and cohesin domains. The DNA encoding the HSPβ-coh fusion construct was inserted into a pET19b expression vector (Novagen). For storage the plasmid was transformed into DH5α cells (Invitrogen). For overexpression of the fusion protein, the pET19b-HSPβ-coh plasmid was transformed into another line of bacteria (*E. coli*) – namely, BL21 CodonPlus (DE3) RIL cells (Stratagene).

In the same manner, PCR-amplified genes for several cellulases (and other lignocellulose deconstruction enzymes) expressed by *C. thermocellum* (ATCC27405DTM) were stored and expressed as described in [19] and [27].

B. Protein Purification and Gel Electrophoresis

Overexpressed proteins including the HSPβ-coh fusion protein and *C. thermocellum* enzymes were purified using a Fast Perfusion Liquid Chromatography (FPLC) system (Pharmacia/GE Amersham). A Superdex 200 size exclusion column and a Mono Q ion exchange column (GE Healthcare) were used to purify desired proteins from lysate. SDS-PAGE and western blot analysis were performed in initial rounds of expression to confirm the purity of protein suspensions and validate molecular weight.

C. mESP complex formation

To induce complex formation of 18 HSP-coh fusion proteins to form an 18-mer double ring enzyme sequestration platform (ESP), mixtures of the fusion proteins at 2 mg/ml were incubated with 1mM ATP and 50mM Mg^{2+} at 4°C for 8-12 hr. [Note that this approach was slightly modified from Mitsuzawa et al. (2009), in which 25mM Mg^{2+} was used]. Complex formation was confirmed using electron microscopy.

D. Transmission Electron Microscopy

Approximately 5µL of protein suspension was spotted onto a formvar-coated copper grid and incubated for 10 min in a humidity chamber. The grid was rinsed with distilled water and negatively stained with 2% (w/v) uranyl acetate for 2 min. The stain was wicked off and the sample was air-dried. Grids were imaged in a Hitachi H-7100 TEM at 75 kV. Images were captured at 60,000–150,000× magnification.

E. mESP charging (with enzymes)

To charge the ESP with cellulose-degrading enzymes, 7.72µM ESP complexes were incubated at room temperature for 15 min. with 6.86µM enzyme or enzyme cocktail in the presence of 0.7mM ATP, 17.4mM $MgCl_2$, and 5mM $CaCl_2$ in 22.2µl of a 50 mM Tris-maleate buffer (pH 6.0).

F. Pretreatment of lignocellulosic feedstock

Field-dried residues were obtained from local farms (Corvallis, OR). Feedstock was milled in a Wiley Mill (Model No. 4, Thomas Scientific, Swedesboro, NJ) equipped with a 2mm round-hole sieve. Ground feedstock was pretreated with dilute acid (1% w/w H_2SO_4) or, alternatively, dilute alkaline solution (1% w/w NaOH) at 10% solid loading at 180ºC for 15 min. All pretreatments were performed in stainless steel tube reactors (316 stainless steel alloy; 19 mm diameter and 305 mm length with capped with Swagelok compression ferrule fittings). The rapid heating of the reactors to the desired temperature was achieved by immersing the reactors in a preheated sand bath (model SBL-2D, 4000 W, Techne Inc., Burlington, NJ) at 270ºC and transferring the tubes to a preheated convection oven to maintain the temperature for the desired residence time. After the reaction time of 15 min, the reactors were immersed in ice-cold water to quench the reactions and bring the reactors to ambient temperature. Heating and cooling times for the reactors were less than four minutes.

G. Sugar Reduction Assays

Enzyme activity (specifically, sugar reduction efficiency) was measured by incubating 0.15% pretreated feedstock with 1.94µl of charged ESP suspension at 65°C for 16h in a 50µl reaction containing: 20mM Tris-maleate (pH 6.0), 1mM ATP, 25mM $MgCl_2$, and, 1mM $CaCl_2$ – followed by a colorimetric assay to assess sugar reduction efficiency. The colorimetric assay [28] compared hydrolytic efficiency between ESP-bound sugar-reducing enzymes versus the same enzymes free in solution (with no ESPs present). After the reaction, samples were centrifuged at 3000 RPM for 10 min. to remove residual particles of the pretreated biomass and diluted 10-fold in sterile DI water. Afterwards, 30µl of the dilute sample was mixed with 30µl of a 50mM Na_2CO_3/10mM KCl solution and 30µl of a 1.5mM $K_3Fe(CN)_6$ solution. This 90µl mixture was heated for 15 min at 99°C and then transferred to a well in a 96-well plate (or a 0.5ml microcentrifuge tube) containing 150ml of a 0.15% $NH_4Fe(SO_4)_2 \cdot 12\ H_2O$ and 0.1% SDS/0.05N H_2SO_4. After incubation at room temperature for 15 min. OD_{690nm} readings were taken using a SpectraMAX M2e (Molecular Devices) automated plate reader (or a Nanodrop spectrophotometer). Both acid-pretreated (H_2SO_4) and alkaline-pretreated (NaOH) lignocellulose feedstock were tested in this manner under both ESP-bound enzyme and free enzyme in solution conditions.

III. Results

HSPβ-coh fusion proteins and enzymes were expressed using a bacterial (*E. coli*) overexpression system. Lysate was purified using FPLC and protein purity and molecular weight verification was confirmed by SDS-PAGE (*data not shown*).

Transmission electron microscopy (TEM) reveals HSP-coh fusion protein complexes (Figure 1). HSP-coh fusion proteins serve as subunits in the formation of a nonameric double-ring (18-mer) ESP complex resembling the size and configuration of natural group II chaperonin heat-shock protein complexes characteristic of Sulfolobus [29], [30].

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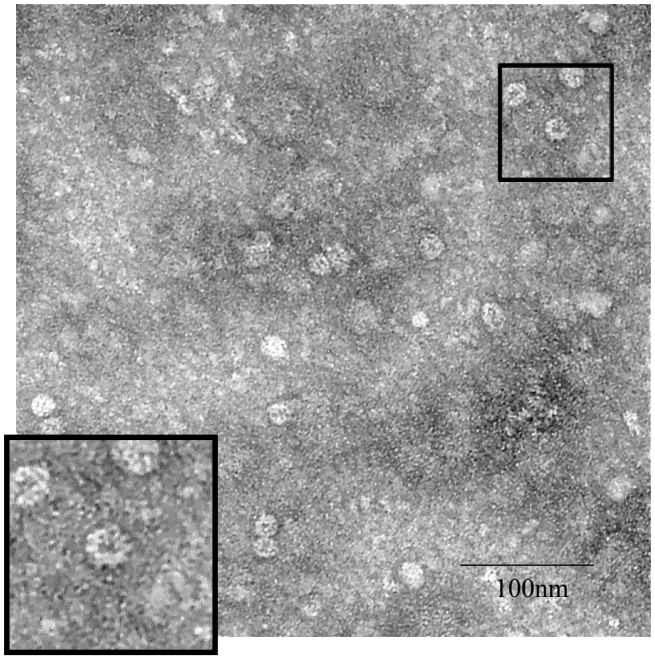

Figure 4 - Transmission electron micrograph of ESP complex. HSP-coh fusion proteins form functional double nonameric ring ESP complexes of approximately 18-22 nm in diameter. Inset shows ring structure with HSP-coh subunits visible.

Using software embedded in the electron microscope system, ESP diameter (18-22nm), when viewing perpendicular to the central pore, is consistent with reports on natural group II chaperonin complexes (also known as thermosomes) [29], [30].

Upon charging these ESPs with lignocelluse degradation enzymes, electron micrographs of the fusion protein complexes become amorphous globular structures under TEM (*data not shown*) due to the attachment of multiple enzymes to a single ESP via cohesin-dockerin interactions (*see* Figure 3).

Using a colorimetric assay [28] to measure the magnitude of hydrolysis during the treatment period, sugar reduction efficiency was assessed for different individual enzymes and for multi-enzyme complements. Two conditions were tested. Enzyme-only treatments consisted of enzymes free in solution without any ESP present. ESP-bound enzyme treatments were also tested on each substrate to determine if the use of ESPs enhanced sugar reduction efficiency.

Enzyme-only systems exhibited similar patterns of sugar reduction activity on both acid-pretreated corn stover (Fig. 5a) and acid-pretreated wheat straw (Fig. 5b). Specifically, the use of xylanase (X), an enzyme that preferentially breaks down hemicellulose [31], showed the greatest hydrolytic activity while CelR, a major processive endoglucanase of the *C. thermocellum* cellulosome [32], showed the least efficiency in reducing acid-treated substrate. Stover and wheat straw hydrolytic efficiency for single enzyme-only systems follows a R<β<S<X trend.

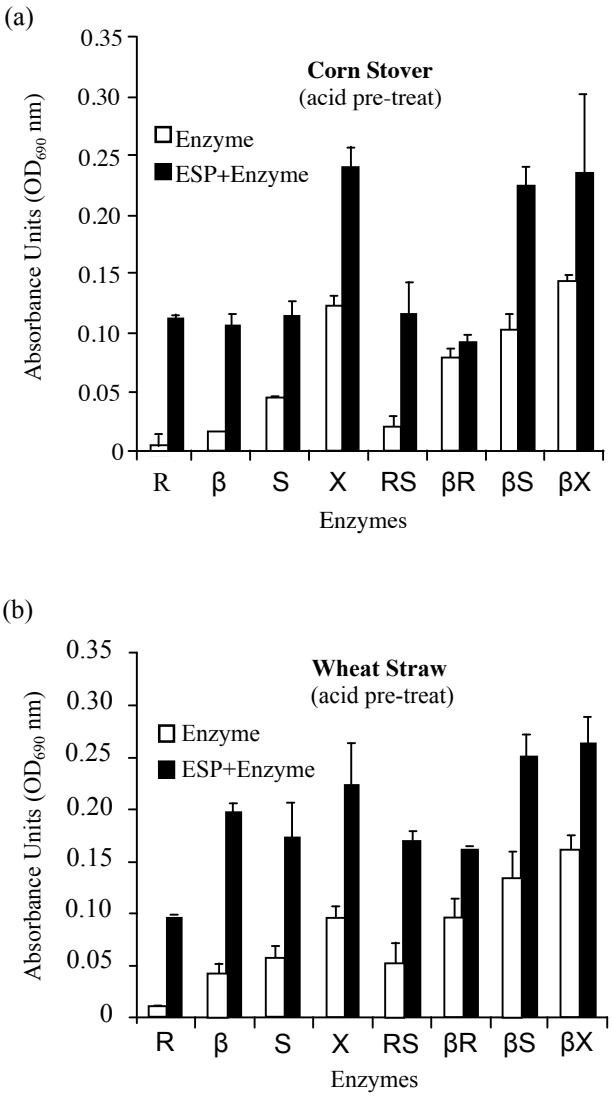

Figure 5 – Sugar reduction on acid-pretreated feedstock. Sulfuric acid (H_2SO_4) pretreated corn stover (*panel A*) and wheat straw (*panel B*) were subject to hydrolysis using enzymes bound to ESPs (black bars) versus free enzymes in solution without ESPs (white bars). *C. thermocellum* enzymes: R – CelR, β – β-glucosidase; S – Cel S, X – xylanase.

With one enzyme type bound to ESP, hydrolytic efficiency significantly increased over the free enzyme-only condition. However, on acid-pretreated corn stover, there was no significant difference between the ESP-bound advantage between ESP-R, ESP-β, and ESP-S constructs. However, the ESP-X system did exhibit a marked increase in sugar reduction efficiency over the other single enzyme charged ESPs for acid-pretreated stover. For single enzyme ESP-bound trials on acid pretreated stover a R≈β≈S<<X pattern of hydrolytic efficiency emerged. For all one enzyme bound cases, at least a two-fold increase in hydrolytic efficiency on acid-pretreated feedstock was observed.

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For two-enzyme systems, hydrolytic efficiency on both acid-pretreated corn stover and acid-pretreated wheat straw exhibited a similar trend for free enzyme-only conditions: RS<βR<βS<βX – even though a statistically significant difference did not emerge between βR vs. βS and βS vs. βX on acid-pretreated wheat straw.

When bound in pairs to ESPs, all two-enzyme complements tested (except for βR on stover) exhibited a significant enhancement of hydrolytic efficiency over the free-enzyme conditions. Although there was no significant difference between ESP-bound advantage using ESP-βS and ESP-βX, each these doubly-bound complements exhibited significantly higher hydrolytic efficiency than the ESP-RS and ESP-βR bound advantage. (Note that there was also no significant difference in ESP-bound advantage between the ESP-RS and ESP-βR constructs). For both acid-pretreated corn stover and acid-pretreated wheat straw, the ESP-bound hydrolytic advantage showed a RS≈βR<<βS≈βX pattern.

As observed in the single-enzyme ESP constructs, several of the two-enzyme ESPs showed about a two-fold increase in sugar reduction efficiency over the free-enzyme cocktail condition. These include: ESP-RS (both stover and straw); and, ESP-βS (stover). In general, these data show that the use of ESP systems on acid-pretreated corn stover and wheat straw result in a significant enhancement of hydrolytic and thus sugar reduction potential.

On alkaline-pretreated substrate, only one single-enzyme system was tested (Fig. 6). β-glucosidase (β) [33], a cellobiose hydrolase, exhibited only modest levels of sugar reduction activity. With β bound to platform, the ESP-β construct showed no appreciable difference in hydrolytic capacity over the free enzyme condition. Similar results were observed in the R-only, S-only, and X-only cases (*data not shown*). However, a few of the enzyme mixes did exhibit significant increases in sugar reduction efficiency on base-pretreated substrates. Furthermore, ESP-bound enzyme complements (e.g., RS and βX) showed significant enhancement in reduction capacity over the free enzyme in solution condition. The alkaline-pretreated substrate trials were limited since focus was on acid-pretreatment (*see* Discussion). It is notable that, in some cases, the magnitudes of ESP advantage in the alkaline-pretreatment trials in some cases were comparable to the acid-pretreatment trials.

Specifically, when β-glucosidase and xylanase are bound to ESP, hydrolytic efficiency over free enzyme in solution ranges from about a 1.4-fold to 1.7-fold increase with lower values observed in the alkaline-pretreatment case (Fig. 6). More variability was observed with the CelR and CelS complement bound to ESP. In alkaline pretreatment trials using ESP-RS, a 2.2-fold and 2.7-fold advantage was observed on wheat straw and corn stover, respectively. However, on acid-pretreated substrates, 3.4-fold and 6-fold ESP advantages were observed for wheat straw and corn stover, respectively (Fig. 5).

Overall, these data show that use of ESPs on feedstock that is alkaline-pretreated can also confer hydrolytic advantage over a free enzyme cocktail.

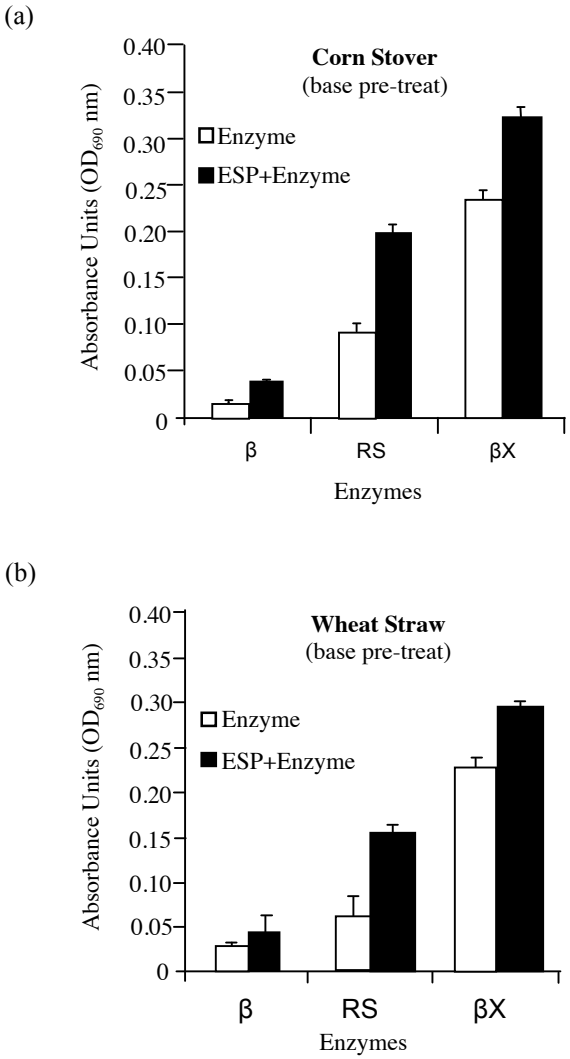

Figure 6 – Sugar reduction on alkaline-pretreated feedstock. Sodium hydroxide (NaOH) pretreated corn stover (*panel A*) and wheat straw (*panel B*) were subject to hydrolysis using enzymes bound to ESPs (black bars) versus free enzymes in solution without ESPs (white bars). *C. thermocellum* enzymes: R – CelR, β – β-glucosidase; S – Cel S, X – xylanase.

IV. Discussion

Feedstock pretreatments typically consist of acid washes or alkaline washes that are designed to initiate deconstruction of lignocellulosic feedstock by inducing swelling in the matrix and interfering with cellulose, hemicellulose, and lignin interactions. After pretreatment, cellulose and hemicellulose are more exposed and susceptible to enzymatic action. Enzyme-mediated deconstruction of the matrix and hydrolysis of complex carbohydrates (e.g., cellulose) yielding simple fermentable sugars (e.g., glucose) in a highly efficient manner can be complicated. The nature of the lignocellulosic material (i.e., the percent composition of cellulose, hemicellulose, lignin, and other components) affects the efficiency of enzymatic action.

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Specific sets of multi-enzyme "cocktails" applied simultaneously or sequentially will have different levels of sugar reduction efficiency on different substrates under different pretreatment conditions. In many processes, pretreatments can leave feedstock slurries with pH and temperature conditions that diminish enzymatic efficiency.

One approach to optimizing enzymatic efficiency is to discover unique enzymes that function under harsh conditions (e.g., low pH, high temperature. An alternative approach is to employ some type of protein scaffold to stabilize enzymes under such adverse conditions. If such platforms are not immobilized, then access to substrate and perfusion through the slurry is not a limiting factor. Here we employed a moblile enzyme sequestration platform (ESP) derived from a hyperthermoacidophilic archaeal heat-shock protein complex. The natural complex, a chaperonin complex, functions to protect proteins within the archaeal cell under conditions of stress (e.g., fluctuations in temperature and pH). Using the ESP, a genetically altered version of this complex, to which cellulases and other lignocellulolytic enzymes can bind, it was hypothesized that enzymatic efficiency could be enhanced on acid-pretreated feedstocks.

To test this hypothesis, acid-pretreated corn stover and wheat straw were subjected to enzymatic action under two conditions. The first condition consisted of having the enzymes free in solution with no ESP utilization. The second condition consisted of using the same enzyme or enzyme complement bound to the ESP.

Electron microscopy demonstrated that HSP-coh fusion proteins form double nonameric ring structures similar to the natural chaperonin complexes from archaea observed *in vitro* [29], [30]. Genetic modification and the resulting two-domain ESP subunit do not inhibit complex formation and apical and basal ends of the complex have exposed cohesin-binding sites. These cohesin binding sites are capable of interacting with any enzyme that contains a *C. thermocellum* dockerin (type I) domain. By charging the ESP with one type or multiple types of enzymes, it was demonstrated that enzymatic efficiency on acid-pretreated corn stover and wheat straw is significantly enhanced. With enzymes selected in this study, an approximate two-fold or greater increase in sugar reduction efficiency was common (with the exception of βR on stover).

Although our hypothesis was initially focused on feedstock that was acid-pretreated due to the fact that the chaperonin proteins from which these constructs were derived are found in hyperthermoacidophilic archaea, we also tested for enhancement of enzymatic efficiency on alkaline-pretreated feedstock, again in a free-enzyme versus ESP-bound enzyme case comparison.

On both acid-pretreated and alkaline-pretreated substrate, xylanase was notably effective at breaking down substrate in single-enzyme treatments. This is likely due to its ability to breakdown xylan, a main constituent of hemicellulose, into xylose, thereby disrupting hemicellulose cross-linking with key matrix components such as lignin [34]-[37]. Charged to the ESP, the efficacy of xylanase enzymatic action increased as much as two-fold on both acid-pretreated stover and straw.

CelR and β-glucosidase exhibited modest hydrolytic efficiency when used alone and free in solution. However, when bound to ESP, a 4- to 6-fold increase in sugar reduction capacity was commonly observed with ESP-R and ESP-β. However, when used in concert as an enzyme complement attached to ESP, ESP-βR on acid-pretreated corn stover showed no significant enhancement and a significant, but modest enhancement on acid-pretreated wheat straw. Furthermore, the overall level of hydrolytic efficiency was lower than ESP-R and ESP-β single-enzyme cases. Thus, no positive synergistic effect was observed. Instead, a "synergistic disadvantage" is apparent and likely due to the distinct modes of action between CelR and β-glucosidase. Whereas, CelR is an endoglucanase that targets internal bonds within the cellulose structure [32], β-glucosidase is an exoglucanase – specifically, a type 2 cellobiose hydrolase, that acts at the terminal non-reducing ends of cellulose to release β-D-glucose [38], [39]. Thus, the two enzymes bound simultaneously to platform may be acting competitively to access internal versus terminal binding sites on cellulose molecules. Also, both CelR and β-glucosidase are processive enzymes, thus a *binding-and-ratcheting* strategy along the molecule can inhibit the other enzyme from effectively hydrolyzing cellulose at the preferred binding site.

Interestingly, the ESP-bound CelR/CelS complement showed a marked (greater than 2-fold) increase in efficiency over the RS enzyme cocktail in both the acid-pretreated and base-pretreated cases on both feedstock types. Since both CelR and CelS are endoglucanases, the ESP-RS construct may allow these two enzymes to act synergistically in attaching to and hydrolyzing cellulose at internal binding sites.

However, the suggestion that charging an ESP with both an endoglucanase and an exoglucanase causes inhibition of one (or the other) enzyme does not necessarily hold. ESP-βS exhibited a significant enhancement in hydrolytic activity over the βS cocktail. A potential explanation for the difference in ESP-βR versus ESP-βS efficacy may reside in the fact that CelR is noted to be more processive than CelS. The ability for CelS to bind, catalyze, and release more readily than CelR may allow for some synergy in the ESP-βS case.

V. Conclusions

Although the nature of synergistic effects using different enzyme complements bound to ESPs need to be elucidated, it is clear from the data presented here that the use of ESPs can significantly enhance hydrolytic efficiency on acid-pretreated (and alkaline-pretreated) feedstock. It is important to note that the use of multiple ESPs, with only one enzyme type charged to each platform remains to be tested. In the present study, ESPs were charged with multiple enzymes (e.g., ESP-RS). Efficiency may be further enhanced by charging only one enzyme class (e.g., endoglucanases), or only one enzyme type (e.g., β), to individual ESPs. Enzyme-charged *platform cocktails* (e.g., ESP-R plus ESP-S) may prove to be more efficient than free enzyme cocktails or ESPs charged with multiple enzymes.

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Furthermore, optimization of sugar reduction efficiency may require enzymes complements for customized for specific pretreated feedstock types. It is also possible that a combination of ESP-bound and free enzymes may provide the most efficient solution to maximizing hydrolytic efficiency. In conclusion, the use of thermo-tolerant and acid-tolerant mobile enzyme sequestration platforms may be one approach to maximizing enzyme-mediated sugar reduction for processes that feature low pH or high temperature conditions.

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