Title

Engineering and Identification of Ionic Liquid-Tolerant Cellulases for Biofuels Production

Permalink

https://escholarship.org/uc/item/4m17q46c

Author

Wolski, Paul William

Publication Date

2013

Peer reviewed|Thesis/dissertation

Engineering and Identification of Ionic Liquid-Tolerant Cellulases for Biofuels Production

By

Paul William Wolski

A

Dissertation submitted in partial satisfaction
of the requirements for the degree of
Doctor of Philosophy
in
Comparative Biochemistry
in the
Graduate Division
of the
University of California, Berkeley

Committee in charge:
Professor Douglas S. Clark, chair
Professor Harvey W. Blanch,
Professor Anastasios Melis
Professor John M. Prausnitz

Fall
2013

Abstract

Engineering and Identification of Ionic Liquid-Tolerant Cellulases for Biofuels Production

by

Paul William Wolski

Doctor of Philosophy in Comparative Biochemistry

University of California, Berkeley

Professor Douglas S. Clark, Chair

Cellulose for biofuels production presents both a great opportunity, in that cellulose is the world's most abundant source of organic material, but also a great challenge in that cellulosic material is highly crystalline and very recalcitrant to degradation. Cellulose is composed of glucose, and this glucose can serve as fermentation feedstock to biofuels in processes that are very well developed. This glucose could also be converted to hydrocarbons similar to diesel for easier commercial adoption.

Before cellulase enzymes can degrade the cellulosic biomass, the biomass generally must be preteated to make the cellulose more accessible to the enzymes (i.e. less crystalline). Typically this involves acid or base treatment that only moderately affects the cellulose. Ionic liquids (ILs), which are organic salts that are liquid at or near room temperature, have the ability to dissolve cellulose by disrupting the hydrogen-bonding network that makes cellulose so strong. Ideally the cellulase enzymes would be active against dissolved cellulose.

The theme of this research has been to combine the pretreatment step with the enzymatic hydrolysis step. The design of the research was as follows: Identify ionic liquids that can dissolve cellulose, while still supporting enzymatic activity and enzymes from nature that can withstand high ionic liquid concentrations. Then, use directed evolution to enhance the ionic liquid tolerance of cellulases and screen for variants that were indeed more IL-tolerant.

Additionally, it was of interest to determine what happens to the enzymes, when inactivated by the ionic liquid. Do they unfold? Does the ionic liquid block the active site?

All of these main objectives were achieved, to varying degrees, in this work. First, using GFP as a reporter protein for quickly measuring protein stability by GFP fluorescence, we identified the ionic liquid 1,3-Dimethylimidazolium dimethylphosphate (Mmim DMP) to support greater

cellulase activity than other ionic liquids, including the more commonly used 1-Ethyl-3-methylimidazolium (Emim) acetate.

Then, we found cellulases from hyperthermophiles, such as *Pyrococcus furiosus*, to be more stable in aqueous ionic liquid than cellulases from mesophilic organisms. A cellulase from this organism was active in up to 70% (w/w) Mmim DMP.

Using DNA shuffling we generated a library of chimeric cellulase (cellobiohydrolase I or Cel7A) genes from several homologous genes. After screening a library of over 1200 variants, we identified two variants that were more stable than the native enzyme from *Talaromyces emersonii*. However, the degree of increase in stability was much less after both the wild type and variant enzyme were treated with exogenous glutamine cyclase to convert the N terminal glutamine to pyroglutamate. This post-translational modification occurs in *T. emersonii*. However, when the wild type and variant enzymes were expressed in *Saccharomyces cerevisiae*, this modification did not occur, and was confirmed via differential scanning calorimetry.

We also used differential scanning calorimetry to determine that the ionic liquid Mmim DMP lowers the melting temperature of the enzyme, in some cases, below the assay temperature. For this reason, we concluded that the ionic liquid, in conjunction with the assay temperature, is working to inactivate the cellulase via global unfolding of the enzyme.

In this work we showed that although enzymatic hydrolysis against dissolved cellulose, was not achieved, we successfully enhanced ionic liquid tolerance of cellulases via directed evolution and by selection of cellulases from extremophiles. We demonstrated a strong correlation between ionic liquid tolerance and thermotolerance. Finally, we confirmed that in directed evolution the winning variants are based directly off the screen used. In this case, evolving cellulases in a system that does not perform the native post-translational modification will not necessarily produce the same results in one that does.

A future study using the same screening system thus should involve either the exogenous addition of the glutamine cyclase enzyme or endogenous production of the glutamine cyclase to make the screen as close to the production of the native host as possible.

Dedication

To my parents William and Eileen Wolski, without whose tireless support from my childhood to today I would not have reached this achievement.

Acknowledgements

        Thank you to Profs. Douglas Clark and Harvey Blanch for your mentorship and support and for this great opportunity. Thank you also to all other Clark lab members for your intellectual and moral support as well. I would also like to thank the Energy Biosciences Institute for generously funding this work. Thank you to Prof. John Prausnitz and Prof. Tasios Melis, who have both provided significant guidance as well during my time here. The Comparative Biochemistry Graduate Program, especially Prof. Fenyong Liu, has been very helpful, and I owe them my thanks for such a supportive environment for my PhD. Inevitably I am missing someone to thank, and I certainly have received and am thankful for all the help from everyone!

Curriculum Vitae

University of California, Berkeley *2009-*
 PhD in Comparative Biochemistry
(expected Dec 2013)
- **Dissertation Thesis:** • Engineering and selecting ionic liquid-tolerant cellulases for biofuels production
- Published in *Green Chemistry* as first author **(2011)** o Presented work at national conferences, including an oral presentation at the American Chemical Society Meeting, 2010 and posters at three national meetings.
- Coinventor for a pending patent on a thermostable cellulase. o Preparing at least one more first author publication.

 Certificate, Management of Technology (College of Engineering and Haas school of Business) *Spring 2013*

Cornell University, Ithaca, NY
2004-2008
 Bachelor of Science in Biological Sciences, concentration: Molecular Cell Biology
2008
(cum laude with distinction in research, dean's list)
- Obtained two separate grants, plus a renewal, to support my independent research on cellulases
- Howard Hughes Scholar
  Summer 2006
- Preparing a first author publication on cellulase binding module pH dependence

RESEARCH AND WORK EXPERIENCE

University of California, Berkeley
 Graduate Student Research, Advisors Douglas S. Clark and Harvey W. Blanch
2009-
- Toward developing a system of enzymatic *in situ* hydrolysis of lignocellulose in aqueous ionic liquids
- Screened a library of over 1200 mutant cellulases for activity in ionic liquids against a solid substrate.
- Characterized library mutant winners and comparison and identified a correlation between thermotolerance and ionic liquid tolerance.
- Determined via differential scanning calorimetry ionic liquid inactivation of the cellulases to be due to global protein unfolding.
- Assayed of several hyperthermophilic archaeal cellulases for activity in high ionic liquid concentrations that had been expressed *cell-free* in *E. coli* cell lysate.

- Studied the effect of post translational modifications on cellulase activity and stability.
- Protein purification using Akta system with and without an affinity tag.
- Used Genbank/Uniprot/KEGG for further analysis and to identify cellulases from halophiles.
- High throughput screen for ionic liquids that can support enzyme activity using GFP shown to be effective for cellulases and for Subtilisin.

Junior Specialist, Research

2008-2009

- Began work engineering ionic liquid tolerant cellulases, developed an original research plan using a high throughput screen for enzyme stability in ionic liquids with a liquid handling robot.
- Studied the effects various ionic liquids on the dissolution of lignocellulosic biomass, including with fluorescence microscopy and infrared microscopy.

Cornell University, Ithaca, NY

2006-2008

Undergraduate Research, Advisor David B. Wilson

- Designed a successful procedure using column chromatography to purify a xylanase (Xyl11A from *Thermobifida fusca*) binding domain. Set up crystalization trials of the protein
- Cloned and expressed this domain in *E. coli*
- Assayed binding of Cel6A to microcrystalline cellulose as a function of pH, was able to explain results using ProPka software.
- Received several grants for undergraduate research including:
  - o Hughes Fellowship
  - o Dextra Grant for Undergraduate Research
  - o Morley Undergraduate Research Grant

Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Summer 2007

Undergraduate Research Program, Advisor Hiroyasu Furukawa

- Expressed NMDA receptor NR2D ligand binding core in a bioreactor from an *E. coli* construct and purified by column chromatography.
- Received training in weekly bioinformatics seminars using databases such as Uniprot, as well as other tools such as BLAST, CLUSTAL, Perl and BioPerl.

Roberts Wesleyan College

Laboratory Technician

2004-2005

- Independently tested experiments prior to their use in a class setting, maintained equipment.
- Helped run a program for science for home-schooled students.

LEADERSHIP ROLES AND ACTIVITIES

University of California, Berkeley
2009-2013
Vice President Energy Biosciences Institute for Berkeley Energy and Resources Collaborative
(BERC)   *2011-2013*
*   Promoted (Bio)energy research and community involvement
*   Organized a panel discussion
Venture Capital Investment Competition
2011-2013

*   Provided biological and expertise that aided two separate teams to reach the final round at UC Berkeley

Mentoring:

Volunteer *2010-2011*
Juma Volunteer tutored underserved high school and college students in science and math
Ventures and served as a role model, helped guide an undergraduate in research who is now pursuing a PhD.

Eagle Scout Lead a large group for a substantial painting project
2003

Publications:

Wolski, PW, DS Clark, and HW Blanch, Green fluorescent protein as a screen for enzymatic activity in ionic liquid–aqueous systems for *in situ* hydrolysis of lignocellulose. *Green Chem*, 2011, 13, 3107-3110.

Publications to be submitted:

Wolski, PW, CM Dana, DS Clark, and HW Blanch, Engineering and identification of ionic liquid-tolerant cellulases for biofuels production. *In preparation for submission.*

Wolski, PW, DC Irwin, DB Wilson, Effect of pH on the binding of the *Thermobifida fusca* Cel6A. *To be prepared.*

Presentations:

Oral:

Wolski, PW, KE Shill, HW Blanch, and DS Clark. Enzymatic hydrolysis of lignocellulose in ionic liquid-aqueous systems. 240th American Chemical Society National Meeting, Boston, MA, Aug 23, 2010.

Posters:

Paul Wolski, Craig M. Dana, Douglas S. Clark, and Harvey W. Blanch. Engineering ionic liquid tolerant cellulase enzymes. 35st Symposium on Fuels and Chemicals Society of Industrial Microbiology, Portland, OR April 29, 2013.

      Also presented at the BERC Innovation Expo. October 10, 2013

Paul Wolski, Craig Dana, Douglas Clark, Harvey Blanch. Engineering ionic liquid tolerant cellulase enzymes. BERC Innovation Expo. October 18, 2012.

Paul Wolski, Maria Francisco De Casal, Douglas Clark, Harvey Blanch. Enzymatic hydrolysis of Lignocellulose in Ionic Liquid Aqueous Systems. BERC Innovation Expo. October 20, 2011.

P. Wolski, S. Padmanabhan, J. Pruasnitz, D.S. Clark, and H. Blanch. Activity and Function of Ionic Liquids for Lignocellulose Dissolution and Hydrolysis. 31st Symposium on Fuels and Chemicals Society of Industrial Microbiology, San Francisco, CA May 3, 2009.

      Also Presented July 8, 2009 at: Biochemical Engineering XVI conference, Burlington, VT.

Introduction

The effect of enzymes, virtually all of which have evolved to work in an aqueous environment, in a nonaqueous system is of interest for a variety of reasons, including enhanced solubility of reagents.[1]   However, a nonaqueous environment can often have a deleterious effect on the enzymes in terms of both stability and activity, and this effect is often determined by the amount of water present in the system as Clark discusses.[2]

In 1985 Zaks and Klibanov contributed to this field by testing lipases in several organic solvents including hexane.[3] The authors' main hypothesis of their study was that the enzymes are proteins that are evolved to work in an aqueous environment, but how much water really was necessary was (and still is in many cases) uncertain. They postulated that the enzyme might need to interact with only one or two layers of water around it and that the composition of the remaining solvent was less important.

Ionic liquids, salts that are liquid at or near room temperature, are another class of solvents that can have unique properties compared to water. This may lead to a desire to perform enzyme reactions in these environments.   For example, ionic liquids have been demonstrated by Swatloski and colleagues in 2002 to dissolve cellulose.[4] Cellulose is a polymer of glucose with a glucose dimer (cellobiose) repeating unit and is the most ambundant polymer on Earth.[5] Cellulose has its crystallinity due to a complex intrachain and interchain hydrogen bonding network.[6]

Swatloski demonstrated the ionic liquid 1-Butyl, 3-methylimidazolium (BMIM) chloride was able to dissolve cellulose.[4] The hydrogen bond accepting anion is able to break up the hydrogen-bonding network of the cellulose, solvating it. Thus for the same reasons that enzyme reactions are carried out in organic solvents (solubility of the reagents) an enzyme reaction might be desirable in such an ionic liquid against a dissolved substrate, such as cellulose, which in water normally has a crystalline form.

The current use of ionic liquids with cellulosic biomass is to make "regenerated cellulose," which is considerably less crystalline. Dadi, et. al.[7] demonstrated that ionic liquid-regenerated cellulose could be much rapidly degraded. In this case ionic liquids could be of great use in replacing acid/base pretreatment currently employed as a preliminary step to convert cellulosic biomass to biofuels. In 2007, Kilpeläinen and colleagues demonstrated imidazolium chloride ionic liquids could dissolve wood.[8] In 2009 Lee, et. al.[9] demonstrated that the ionic liquid 1-Ethyl, 3-methylimidazolium (EMIM) acetate also was able to dissolve whole biomass.

One example of a reaction (especially one that does not include a lipase) in a nearly pure ionic liquid environment would be the synthesis of lactose from glucose and galactose using the enzyme Beta galactosidase from *Bacillus circulans*, as reported by Kaftzik and colleagues in 2003.[10] In this study the reaction is put "in reverse" of its typical reaction pathway, forming a glycosidic bond instead of breaking it. Nevertheless, here the enzyme performs best of the ionic liquids tested in 1,3 Dimethylimidazolium methylsulfate, giving a 17% product yield after 24 hrs in a system that only contained 0.6% water by volume. In this case there is no other liquid solvent/reagent in which the enzyme could reside for stability.

Another, perhaps more common, reaction medium including ionic liquids is one with a significant amount of water present in a single phase. As 1-Butyl, 3-methylimidazolium chloride (BMIM Cl) was the first ionic liquid discovered to dissolve cellulose, it made sense to study the activity of cellulases in such conditions. Cellulases are the enzymes that degrade cellulose and work in concert with a β-glucosidase, an enzyme that cleaves cellobiose to glucose.[11] In 2003, Turner and colleagues[12] tested the cellulase from *Trichoderma reesei* in pure BMIM Cl, but did

not observe any enzymatic activity. Even 22 mM BMIM Cl was enough to slow down the enzyme. (An activity loss at such a low ionic liquid concentration may be the case in studies with other enzymes/ionic liquids, but the resolution of the assays in those experiments may be insufficient to detect such changes in activity). The authors then wanted to see how much BMIM Cl would *unfold* the enzyme. Fluorescence assays based on what is likely tryptophan fluorescence showed the enzyme going through an intermediate denaturation state at 10% ionic liquid concentration and then fully unfolded at 20% BMIM Cl. The authors then tried to stabilize the enzyme by binding it to a PEG 1000 support. They tested this enzyme with PEG only in neat BMIM Cl, and observed a marginal increase in activity only.

In another study, by Kamiya et. al in 2008,[13] a different ionic liquid, 1-Ethyl, 3-methylimidazolium diethylphosphate, was chosen based on previous information from Fukaya et al in 2008[14] about the ability of similar ionic liquids being able to dissolve cellulose. After dissolving cellulose in the above ionic liquid, a buffer at the optimal pH of the cellulase was added. They observed after 24 hrs greater conversion of cellulose to glucose in 20% ionic liquid than in an aqueous buffer alone and equal conversion in 40% to the aqueous buffer. These results give promise to potential ability to hydrolyze cellulose in the presence of ionic liquid after dissolution without first removing the ionic liquid completely. The cellulose reformed insoluble "regenerated cellulose" after addition of the aqueous buffer. This regenerated cellulose is almost certainly more easily hydrolysable than crystalline cellulose.[7] However the authors appear to use the crystalline cellulose as the substrate with no ionic liquid present as the means for comparison of enzymatic rates between an aqueous-ionic liquid system and purely aqueous system. A better study would be to use washed "regenerated cellulose" as the substrate in the purely aqueous environment as a better means of comparison, and what would almost certainly be seen is that, at least for the 40% ionic liquid environment, enzymatic rates would actually be lower than in aqueous. However, this paper identifies one ionic liquid that had heretofore shown the best enzymatic hydrolysis of cellulose using native *Trichoderma reesei* cellulases.

Given the disparity between performance of the same enzyme (*Trichoderma reesei* cellulase) in different ionic liquids, as described in two of the reports above, one might ask, "What inherently makes one ionic liquid better for enzymes than another?" There have been several attempts to answer this question, nearly all of which center around the concept of the Hofmeister series, which is based on the general concept that certain ions will either bring about the exposure or burial of the normally-internal hydrophobic regions of proteins due to changes in the structuring of the water around the surfaces of the protein. The Hofmeister series is generally empirically determined, but has some basis in the charge density of the ions. Constantinescu and colleagues in 2007 performed an analysis of several ionic liquid cations and anions in the context of the Hofmeister series to try to determine which ions are best for protein stability.[15] Their results at least seem to correlate with the reaction rate results determined by others that sulfate and phosphate based anions are superior to chloride anions for proteins, fitting the general idea, which they also demonstrate, that kosmotropic (generally high charge density) anions and chaotropic (typically low charge density) cations are better for protein stability. They note that, with more complex ions it is much more difficult to predict exactly what kind of effect a particular ion will have relative to other ions that are very similar.

A stronger motivation for studying cellulase enzymes is that fact that cellulose is the largest source of organic material on the planet.[5] It has been estimated that cellulosic biofuels feasibly could replace 1/3 of US fossil fuel consumption.[16] One of the bottlenecks of cellulosic biofuels production has been the crystallinity of the substrate. Ionic liquids, as mentioned, can

ablate this crystallinity. Enzymatic hydrolysis of lignocellulose dissolved in ionic liquid could lead to much faster hydrolysis[17] (even over regenerated cellulose) due to zero crystallinity of the substrate, leading to access to all of the substrate at once by the enzymes. Additionally, there could be industrial advantages of the reduced process steps by combining pretreatment and hydrolysis, and it is therefore of interest to identify and develop cellulases that can tolerate an ionic liquid environment.

References:

1. R. P. Swatloski, S. K. Spear, J. D. Holbrey and R. D. Rogers, *J Am Chem Soc*, 2002, **124**, 4974-4975.
2. D. S. Clark, *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, 2004, **359**, 1299-1307.
3. A. Zaks and A. M. Klibanov, *Proc Natl Acad Sci U S A*, 1985, **82**, 3192-3196.
4. R. P. Swatloski, A. E. Visser, W. M. Reichert, G. A. Broker, L. M. Farina, J. D. Holbrey and R. D. Rogers, *Green Chem*, 2002, **4**, 81-87.
5. E. A. Bayer, H. Chanzy, R. Lamed and Y. Shoham, *Current Opinion in Structural Biology*, 1998, **8**, 548-557.
6. R. Parthasarathi, G. Bellesia, S. P. S. Chundawat, B. E. Dale, P. Langan and S. Gnanakaran, *The Journal of Physical Chemistry A*, 2011, **115**, 14191-14202.
7. A. P. Dadi, C. A. Schall and S. Varanasi, *Appl Biochem Biotechnol*, 2007, **137-140**, 407-421.
8. I. Kilpeläinen, H. Xie, A. King, M. Granstrom, S. Heikkinen and D. S. Argyropoulos, *Journal of Agricultural and Food Chemistry*, 2007, **55**, 9142-9148.
9. S. H. Lee, T. V. Doherty, R. J. Linhardt and J. S. Dordick, *Biotechnol Bioeng*, 2009, **102**, 1368-1376.
10. N. Kaftzik, S. Neumann, M. Kula and U. Kragl, in *Ionic Liquids as Green Solvents: Progress and Prospects*, eds. R. D. Rogers and K. R. Seddon, ACS Symposium Series, 2003, vol. 856, p. 206.
11. D. B. Wilson, *Annals of the New York Academy of Sciences*, 2008, **1125**, 289-297.
12. M. B. Turner, S. K. Spear, J. G. Huddleston, J. D. Holbrey and R. D. Rogers, *Green Chem*, 2003, **5**, 443-447.
13. N. Kamiya, Y. Matsushita, M. Hanaki, K. Nakashima, M. Narita, M. Goto and H. Takahashi, *Biotechnol Lett*, 2008, **30**, 1037-1040.
14. Y. Fukaya, K. Hayashi, M. Wada and H. Ohno, *Green Chem*, 2008, **10**, 44-46.
15. D. Constantinescu, H. Weingartner and C. Herrmann, *Angew Chem Int Edit*, 2007, **46**, 8887-8889.
16. S. N. Laboratories, ed. S. N. Laboratories, US Department of Energy Publications, 2009, vol. 84.
17. T. Jeoh, C. I. Ishizawa, M. F. Davis, M. E. Himmel, W. S. Adney and D. K. Johnson, *Biotechnol Bioeng*, 2007, **98**, 112-122.

Chapter 1
Green fluorescent protein as a screen for enzymatic activity
in ionic liquid–aqueous systems for *in situ* hydrolysis of
lignocellulose

Abstract:

A high throughput screen was developed to test the stability of proteins in ionic liquid-aqueous mixtures using Green Fluorescent Protein (GFP) as a reporter. In some ILs, over 50% of GFP fluorescence was maintained in an IL/aqueous mixture with IL concentrations as high as 75%. Using this screen, we identified 1,3 Dimethylimidazolium dimethylphosphate that supports cellulolytic activity of an unmodified *Trichoderma reesei* cellulase cocktail in IL concentrations as high as 40 weight % against *Miscanthus* fibers that were previously dissolved in the same IL. Further characterization of the ionic liquid's effect on biomass-degrading enzymes revealed β-glucosidase stability in this IL in concentrations as high as 60%.   The protease subtilisin also maintained activity in 45% (w/w) Mmim DMP, confirming the utility of GFP as a reporter for potential enzyme function in ILs.

Green Fluorescent Protein as a Screen for Enzymatic Activity in Ionic Liquid-Aqueous Systems for *in situ* Hydrolysis of Lignocellulose

A rapid screen was developed to test the stability of proteins in ionic liquid-aqueous mixtures using green fluorescent protein (GFP) as a reporter. In at least one ionic liquid (IL), GFP retained 50% or more of its fluorescence in IL volume fractions as high as 75%. ILs that best preserved GFP fluorescence also showed the best retention of cellulase activity. Using this screen, two potential candidates for *in situ* enzymatic hydrolysis of biomass, 1,3-dimethylimidazolium dimethylphosphate (Mmim DMP) and 1-ethyl-3-methylimidazolium (Emim) lactate, were identified. A commercial *Trichoderma reesei* cellulase mixture retained activity in both ILs up to 40% (w/w) IL, and β-glucosidase remained active after incubation in 60% (w/w) Mmim DMP for 8 hours, indicating the possibility of *in situ* cellulose hydrolysis in IL/water mixtures.

Introduction

Ionic liquids (ILs) were reported by Rogers and coworkers in 2002 to dissolve cellulose; in particular, the IL 1-butyl-3-methylimidazolium (Bmim) chloride was shown to dissolve up to 10 % (w/w) cellulose (25% (w/w) with microwaving).[1] The ability of ILs to dissolve lignocellulose raises the possibility of enzymatic hydrolysis of its cellulosic content *in situ*. In this work, we provide a rapid screen for the stability of proteins in IL-water mixtures as a means to assess the feasibility of *in situ* enzymatic cellulose hydrolysis. The fluorescence of Green Fluorescent Protein (GFP) was employed as an indicator of protein unfolding in IL-water mixtures to identify ILs that allowed retention of enzyme activity.

The mechanism of cellulose dissolution in ILs was proposed to involve disruption of hydrogen bonds within the cellulose by the anion. Kilpeläinen et al. subsequently demonstrated imidazolium chloride ILs could dissolve wood,[2] and later Lee et al.[3] demonstrated that 1-ethyl-3-methylimidazolium (Emim) acetate was also able to dissolve lignocellulosic biomass. Cellulose or the cellulosic component of lignocellulosic biomass can be precipitated from ILs by the addition of an anti-solvent such as water.[1] Dadi et al. demonstrated that IL-regenerated cellulose could be rapidly enzymatically degraded by commercial cellulase mixtures.[4] IL dissolution could thus provide an alternative to the dilute acid pretreatment step typically employed to improve the conversion of lignocellulosic biomass to sugars for subsequent fermentation to biofuels.

In situ enzymatic hydrolysis of cellulose dissolved in ILs could provide a facile route to produce glucose and xylose sugars from dissolved biomass. The ability of enzymes to function in ILs has been examined with the aim of minimizing the water activity in the reaction.[5] A relevant example is the synthesis of lactose from glucose and galactose by β-galactosidase, which gave a 17% yield after 24 h in 1,3-dimethylimidazolium (Mmim) methylsulfate with 0.6% (v/v) water.[6] However, cellulases from *Trichoderma reesei* were found to be inactive in 95% Bmim Cl,[7] and their activity in an aqueous solution containing 22 mM Bmim Cl was reduced. Based on tryptophan fluorescence, the cellulases unfolded at concentrations higher than 20% (w/w) Bmim Cl.

Enzymatic activity has also been observed in other ILs. Crystalline cellulose (Avicel) was dissolved in Emim diethylphosphate and precipitated by the addition of an aqueous buffer

solution containing cellulase.[8] Enzymatic hydrolysis of the precipitated cellulose in a 20 % (v/v) Emim diethylphosphate-water mixture yielded 70% conversion to sugars after 24 hours. Cellulose precipitated after dissolution in ILs is more easily hydrolyzed than crystalline cellulose, as the cellulose is decrystallized[1] and hydrolysis rates are related to the degree of crystallinity of the substrate.[9]

The Hofmeister series provides insight in determining which ions might enhance protein stability in ILs.[10] The unfolding temperature of ribonuclease A in various IL-aqueous mixtures was observed by differential scanning calorimetry and was found to correlate with the Hofmeister series. This has previously been shown for salts other than ILs.[11] However, *a priori* prediction of protein stability in ILs remains unreliable.[10] Thus, the objective of the present work was to develop a rapid screen for enzyme stability and activity in a variety of aqueous-IL solutions.

GFP fluorescence has been used as a reporter for protein unfolding.[12,13] Heller et al. observed GFP fluorescence in Bmim chloride in 25 and 50% (w/w) aqueous solutions at 20, 40, and 60ºC. Small angle neutron scattering of the protein was employed to observe unfolding and aggregation, and was shown to correlate with the green fluorescence of the protein.[14] Li and colleagues[15] found Mmim dimethylphosphate (DMP) to be the best IL for maintaining cellulase activity among the ILs tested, and this is confirmed in the present work.

Results and Discussion

GFP fluorescence was observed in mixtures of various ILs and aqueous buffer (50 mM Tris, 150 mM NaCl pH 7.6). In the IL-aqueous buffer mixtures that reduced GFP fluorescence, the mixture pH was between 7.4 and 8.2. Over this pH range, the fluorescence of GFP does not change significantly.[16,17] Loss of fluorescence indicates that GFP has unfolded.[13] All of the ILs studied here are able to dissolve lignocellulosic biomass and cellulose (Avicel). The presence of ILs attenuates the GFP fluorescence, without shifting the wavelength of maximum emissivity, and fluorescence was measured at 535 nm after excitation at 485 nm. The results shown in Figure 1 indicate that GFP retains its ability to fluoresce in Mmim DMP at an IL concentration of 75% (v/v). In two other ILs, Emim ethylsulfate and 2-hydroxyethyl-trimethylammonium lactate, GFP fluorescence was maintained in IL concentrations up to 85% (v/v). However, these ILs are not able to dissolve significant amounts of cellulose or lignocellulose.

The results of the GFP screen were compared with those based on the catalytic activity of a cellulase mixture (Celluclast 1.5L and Novozyme 188). The results indicate that the ILs that preserved GFP fluorescence also showed the best retention of cellulase activity (Figure 2). In Emim lactate and Mmim DMP, complete conversion of Avicel to glucose was observed after 16 h in 10 and 20% (w/w) IL mixtures, respectively. In 40% (w/w) Mmim DMP, 21% conversion to glucose was observed; in 40% (w/w) Emim lactate, the corresponding conversion was 16%. The IL concentrations (v/v) resulting in a 50% reduction in GFP fluorescence correlated linearly with the interpolated IL concentrations (w/w) resulting in cellulase conversion of 50% of the cellulose.

Figure 3 illustrates the activity of β-glucosidase on cellobiose in aqueous mixtures of Mmim DMP or Emim acetate, with and without pH adjustment. Nearly complete conversion of cellobiose to glucose was observed in Mmim DMP from 0-30% (w/w) IL at pH 4.8 and in solutions where the pH was not adjusted. A reduction in activity was observed at 40% (w/w) Mmim DMP, and in 19% (w/w) Emim acetate aqueous mixtures. The pH values of IL/water mixtures that were not adjusted to 4.8 are given in Table 1. β-glucosidase is active between pH 3.2 and 4.8 and less active at pH 6.3 and higher.

To further examine enzyme stability in ILs, β-glucosidase was incubated in IL-water mixtures at 50°C for 28 h. Cellobiose was then added and conversion to glucose was measured after 3 hours. Complete hydrolysis of cellobiose to glucose was observed in aqueous mixtures of 0, 10, and 20 % (w/w) Mmim DMP (Figure 4). However, activity was completely lost after 28 h in 40% Mmim DMP and in 19% Emim acetate. Incomplete conversion was observed in 30% (w/w) Mmim DMP and in 10% (w/w) Emim acetate. These results indicate that β-glucosidase is initially active in 40% Mmim DMP and 19% Emim acetate, but it does not remain active at these IL concentrations after 28 h at 50 °C.

The data in Figures 1, 3, and 4 (all of which correspond to a single enzyme/protein) were fit to a sigmoidal form. The sharp decrease in fluorescence or activity indicates the IL concentration at which the protein unfolds in what appears to be a single-stage process.

The reversibility of β-glucosidase inactivation was examined in 50% (w/w) Mmim DMP at pH 4.8 as function of incubation time and temperature. The enzyme was incubated without substrate in 50% (w/w) Mmim DMP, pH 4.8, for 1-8 h at the temperatures indicated in Figure 5, and the mixture was then diluted 10-fold. Substrate was added to a final concentration of 0.1% (w/w) and enzyme activity was assayed at room temperature. In 50% (w/w) Mmim DMP, no significant conversion of cellobiose to glucose was observed at temperatures between 20 and 50°C; however, activity was recovered upon ten-fold dilution of the IL except for the samples that had been incubated at 50°C. The enzyme also retained activity when incubated in 60% (w/w) Mmim DMP, pH .8, at 30°C for 1-8 h and then diluted 10-fold. These results reflect a synergistic effect between temperature and IL concentration for enzyme stability that produces an apparent lowering of the inactivation temperature of the enzyme when incubated in a higher IL concentration.

Figure 6 shows cellulase activity against *Miscanthus* that had been dissolved in Mmim DMP and Emim acetate at 140°C for 18 h. Enzyme activity was observed in Emim acetate at concentrations up to 19% (w/w) and in Mmim DMP at concentrations up to 40% (w/w), which are both also the highest IL concentrations in which cellulase activity was observed against IL-pretreated Avicel. Complete conversion of the cellulosic content of the biomass to glucose was not observed, even in the absence of IL, which was likely due to suboptimal pretreatment conditions. The protease subtilisin showed activity in the same ILs at various concentrations. Proteolytic activity was retained in Mmim DMP up to 45% (w/w) IL, whereas lower concentrations of Emim lactate, Emim acetate, and Bmim acetate were required for retention of subtilisin activity. These results are shown in Figure 7.

Conclusions

In this work, we demonstrated the utility of using green fluorescent protein in a screen for retention of enzyme activity in various ILs. Using this screen, two potential candidates for *in situ* enzymatic hydrolysis of biomass, Mmim DMP and Emim lactate, were identified.

Materials and Methods:

GFP production: *E. coli*
BL21 DE3 containing the gene for E GFP (BD Biosciences) in a pET19 vector (Invitrogen) was grown in Terrific Broth. After cell lysis, the soluble fraction was loaded onto Ni-NTA and eluted with imidazole. The imidazole was dialyzed with 50mM Tris, 150 mM NaCl, pH 7.6.

Fluorescence measurements of GFP in ILs:

ILs (Iolitec or Sigma-Aldrich) and/or 50 mM Tris,150 mM NaCl pH 7.6 were pipetted into a 96-well black plate using a Biomek FXP robot (Beckman-Coulter). GFP solution (20.0 µl, 0.5 mg/ml) was then added, bringing the volume to 200 µl. The plate was then shaken for 30 min, which Alkaabi and colleagues showed to be sufficient time for loss of GFP fluorescence under denaturing conditions,[18] and the fluorescence was measured with excitation at 485 nm and emission at 535 nm using a Beckman Coulter DTX 880 Multimode detector.

Cellulase activity against Avicel:

Avicel (3% moisture) (Sigma-Aldrich) was added to ILs, and each reaction tube was vortexed and then agitated for 24 h at 60°C to dissolve the cellulose. Deionized water was then added to the samples prior to subsequent agitation overnight. The pH was adjusted as required. β-Glucosidase [Novozyme 188, 208 units/ml] and *Trichoderma reesei* cellulase [Celluclast 1.5L, 130 FPU/ml], both from Novozymes, were then added to each reaction mixture. The cellulase was added at a concentration of 20 FPU/g substrate. The β-glucosidase loading, 46.8 units/g cellulose, was in excess based on information from the supplier. The tubes were rotated end-over-end at 25 rpm for 16 h at 50°C. Then, 1.0 ml of the reaction was added to 9.0 ml of distilled water and mixed. The glucose content was determined with a YSI (Yellow Springs Instruments) glucose analyzer.

β-Glucosidase initial activity:

46.8 units *β-glucosidase*/g cellobiose was added to the IL samples with cellobiose and assayed using the same method as for cellulase activity toward Avicel.

β-Glucosidase incubation:

Samples at various IL concentrations without substrate were adjusted to pH 4.8. β-Glucosidase was then added at the same loading as above and incubated at various IL concentrations and temperatures before addition of cellobiose.

Cellulase activity toward Miscanthus:

Miscanthus giganteus (University of Illinois, Urbana-Champaign) was ground to a sieve size of 0.08 mm. The moisture content was 8%, and the cellulose content 40% (dry basis). The *Miscanthus* was dissolved at 1.0% (w/w) cellulose loading in the ILs for 18 h at 140°C with stirring. The cellulose (from the *Miscanthus*) loading in each enzymatic reaction was 0.10% (w/w). The samples were adjusted to pH 4.8 and diluted with varying amounts of water, and the cellulose conversion to glucose after 16 h at 50 °C was determined at the same enzyme loading as employed previously. For hydrolysis reactions in the absence of IL (0% IL), cellulose was precipitated from *Miscanthus* in IL by adding water following dissolution of the *Miscanthus* in the IL. Enzymatic hydrolysis was carried out in 50 mM citrate pH 4.8 at 50°C for 16 h.
Curve fitting for Figures 1, 3, and 4: The data were fit using a sigmoidal function ($a/(b+ce^x)$) as the general form, where a, b, and c are arbitrary constants, e is the exponential constant, and x is the percent IL.

Subtilisin assays:

The protease subtilisin from *Bacillus licheniformis* was obtained from Sigma (# P5380).

Enzyme in reaction was prepared to a final reaction concentration was 97 ng/ml.

The substrate (N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide, Sigma # S7388) was prepared in a 50 mM stock in DMF (dimethylformamide) that was diluted to a final reaction concentration of 452 μM. The reactions proceeded at 25°C for 60 minutes, taking time points every 40 sec. The rate was assayed by the appearance of the yellow p-nitroanilide product with the absorbance at 410 nm.
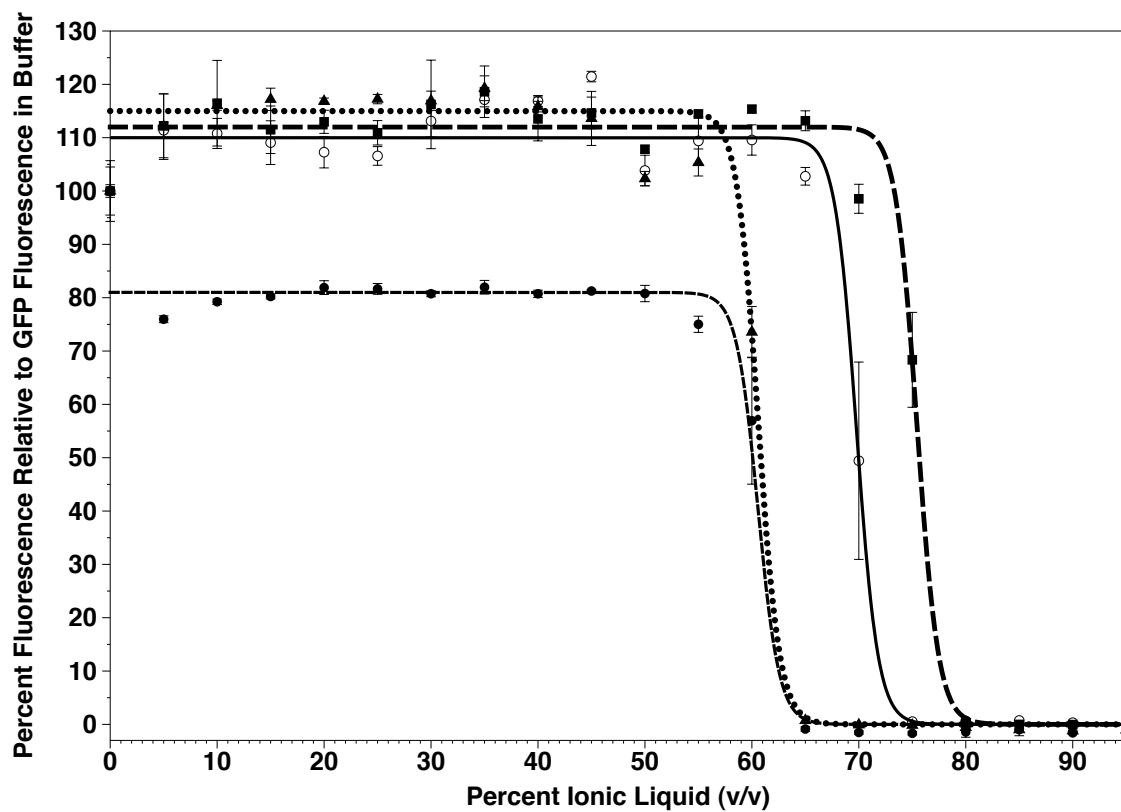
Fig. 1. Green fluorescent protein (GFP) in IL-aqueous solutions. GFP was incubated either in 50 mM Tris, 150 mM NaCl buffer pH 7.6 or in IL-buffer mixtures at the volumetric ratios indicated for 30 min at room temperature and then assayed for fluorescence with excitation at 485 nm and emission at 535 nm. (●) Emim acetate; (▲) Bmim acetate; (■) Mmim DMP; (o) Emim lactate. The curves were fit to a sigmoidal function.
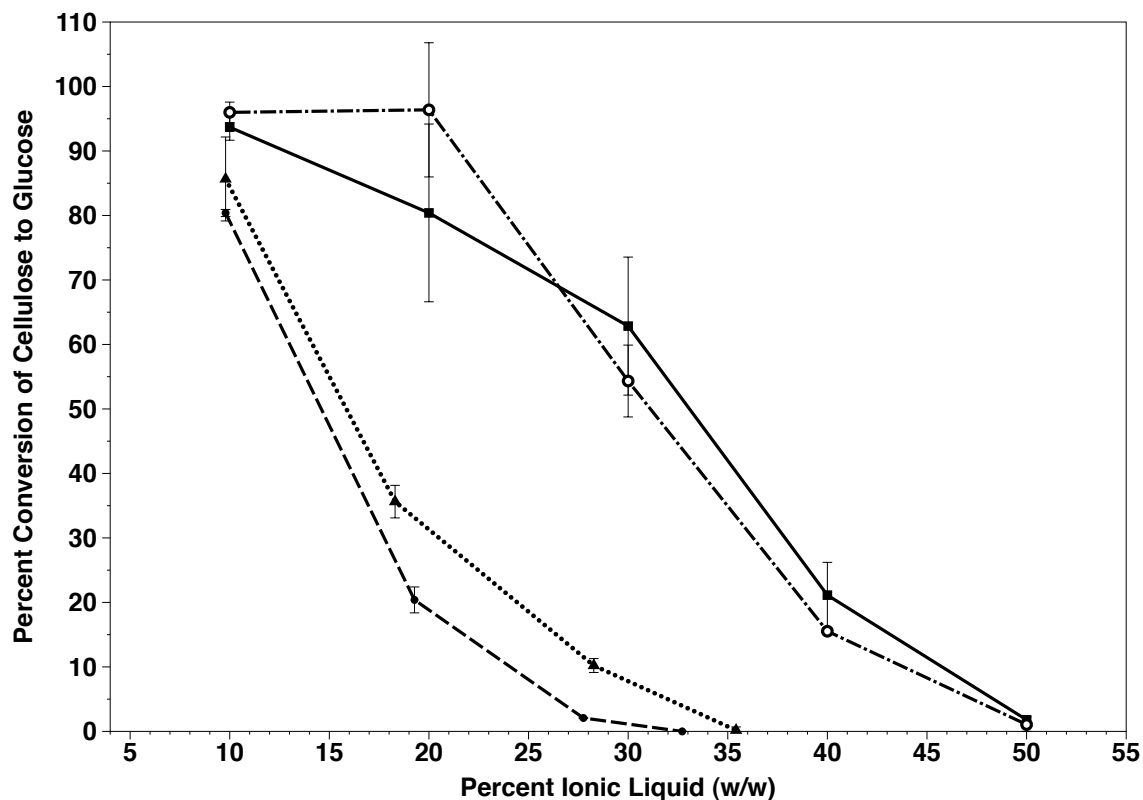
Fig 2. Cellulase and β-glucosidase activity in the presence of IL. Celluclast 1.5L at a loading of 20 FPU/g cellulose and excess β-glucosidase were assayed in mixtures of ILs and water at 50°C, pH 4.8, containing a final Avicel concentration of 1.0% (w/w). Avicel was pretreated by dissolution in each IL at 60°C for 24 h. The pH was adjusted to 4.8 by addition of acetic or citric acid. Conversion of Avicel to glucose is reported at 16 h with a glucose analyzer. (●) Emim acetate; (▲) Bmim acetate; (■) Mmim DMP; (o) Emim lactate. Lines are provided to guide the eye.
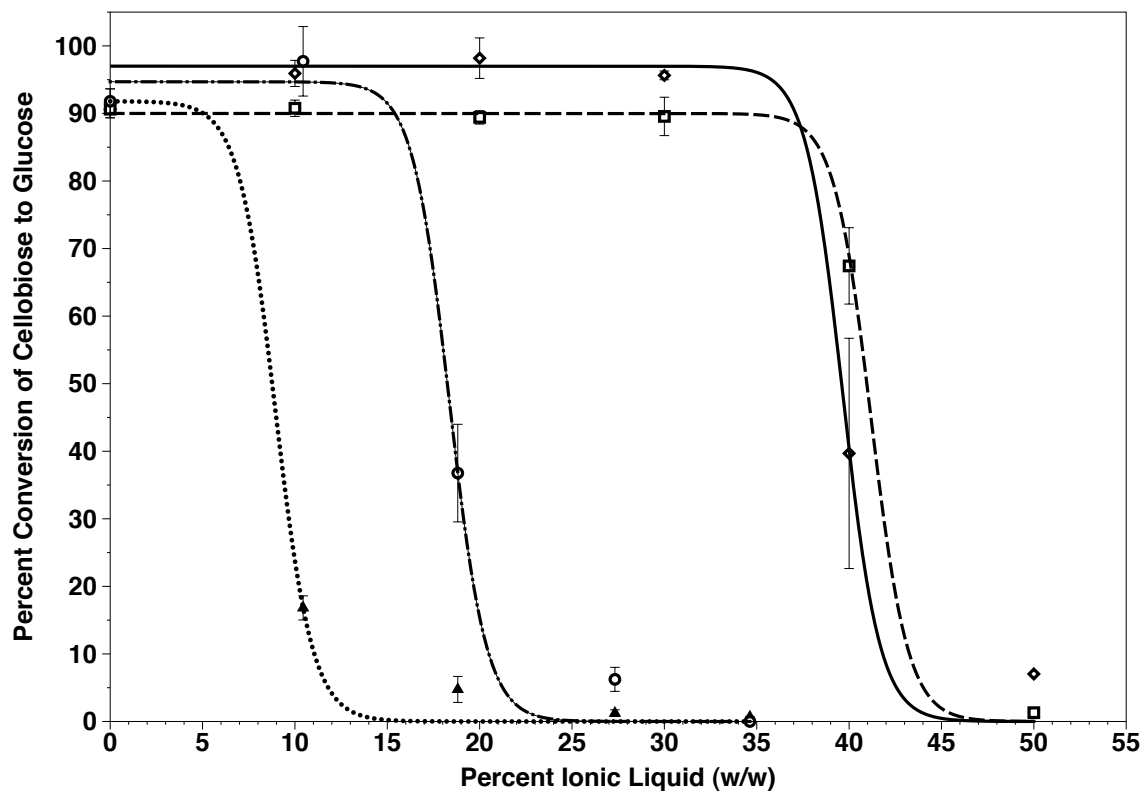
Fig. 3. Effect of pH on β-glucosidase activity in the presence of IL. Excess β-glucosidase was added to diluted IL along with cellobiose (1.0% (w/w) final concentration) with or without pH adjustment (pH values are given in Table 1). The reaction occurred for 16 h at 50°C and was assayed on a glucose analyzer. (◇) Mmim DMP pH 4.8; (□) Mmim DMP; (o) Emim acetate pH 4.8; (▲) Emim acetate. The curves were fit to a sigmoidal function.
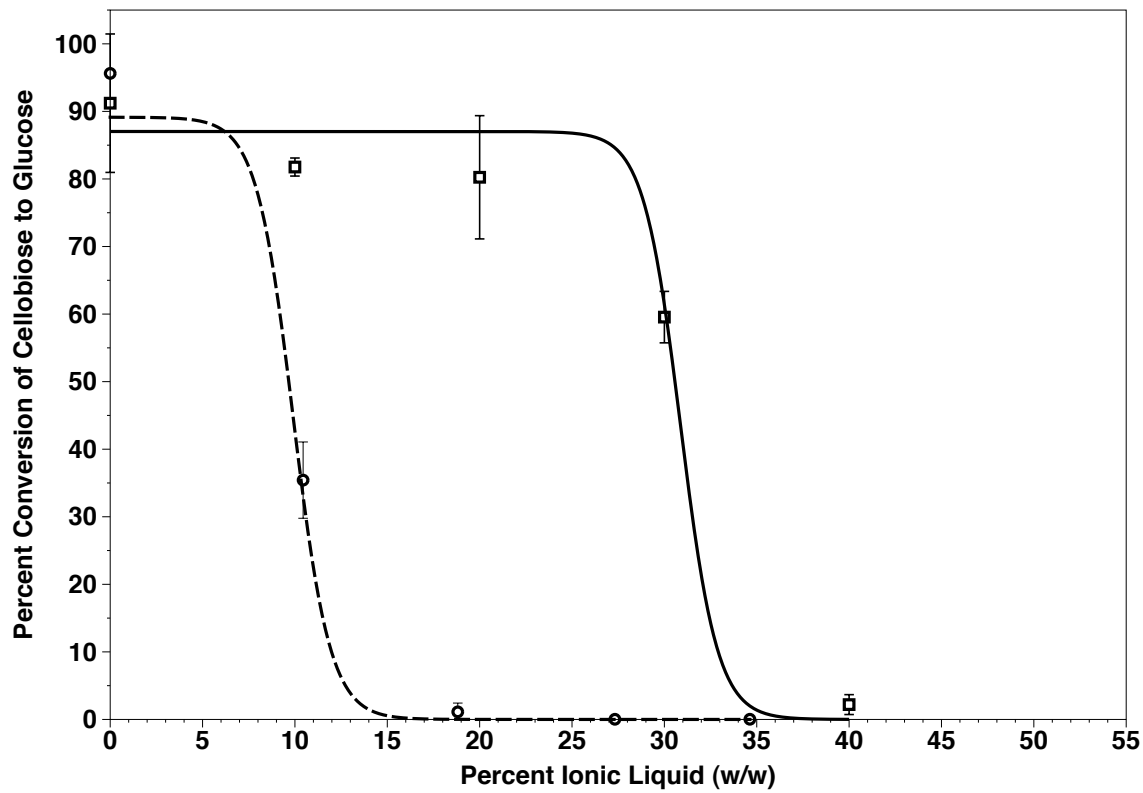
Fig 4. β-Glucosidase activity in the presence of IL after incubation at 50°C for 28 h. Cellobiose was added to 1.0% (w/w). At the end of the enzyme assay, glucose content was determined with a glucose analyzer. ( ) Mmim DMP pH 4.8; (o) Emim acetate pH 4.8. The curves were fit to a sigmoidal function.
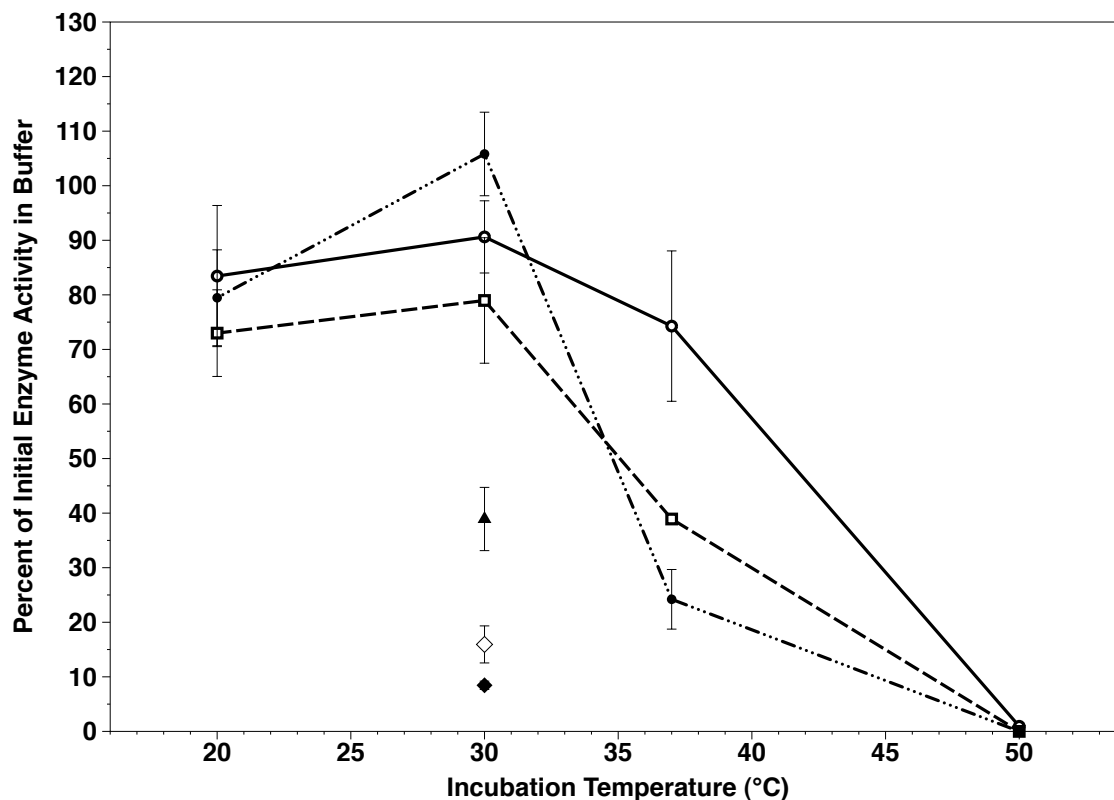
Fig. 5 β-Glucosidase activity after incubation in either 50 or 60% (w/w) Mmim DMP at pH 4.8 and various temperatures. The IL and enzyme were then diluted 10-fold, cellobiose was added, and the conversion rates of cellobiose to glucose were assayed at room temperature with a glucose analyzer. The graph shows β-glucosidase activities relative to the rates at room temperature without incubation in IL. (o) Incubation in 50% Mmim DMP pH 4.8 for 1 h; ( ) Incubation in 50% IL for 4 h; ( ) Incubation in 50% IL for 8 h. (●) Incubation in 60% IL for 1 h; (n) Incubation in 60% IL for 4 h; (♦) Incubation in 60% IL for 8 h. Lines are provided to guide the eye.
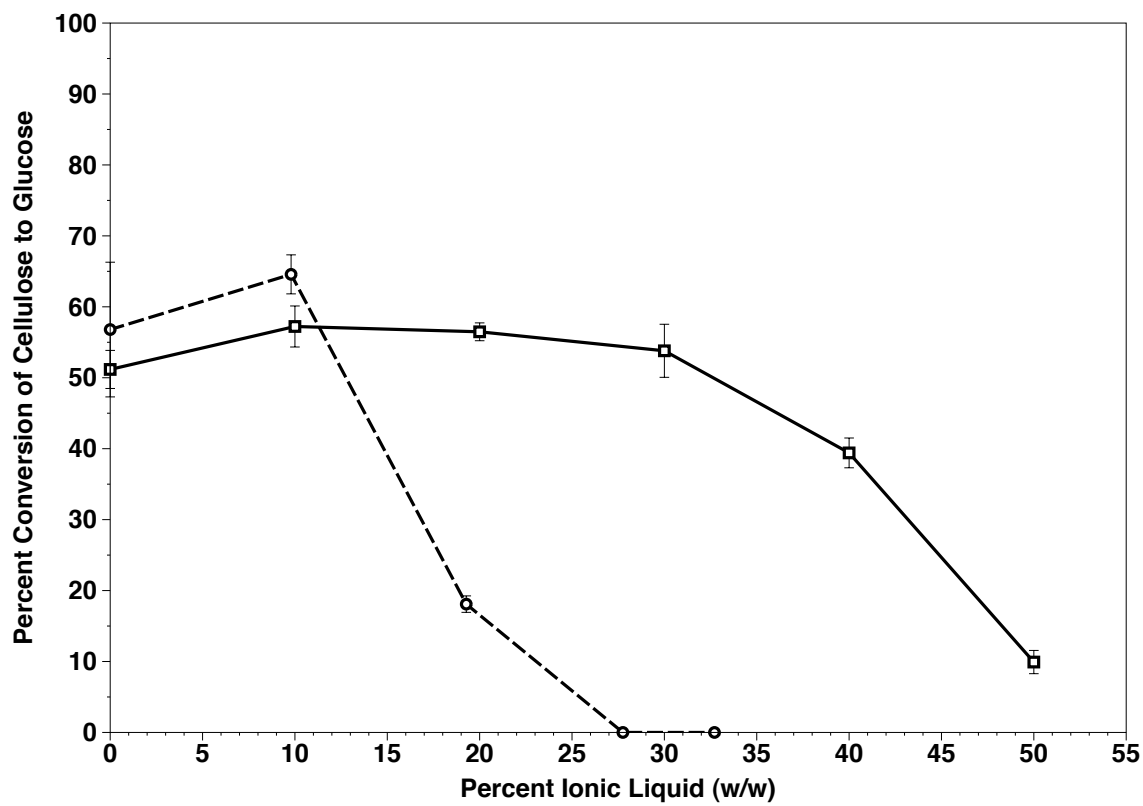
Fig. 6. Cellulase activity toward *Miscanthus.* Celluclast 1.5L (20 FPU/g cellulose) and excess β-glucosidase were added at 50°C to diluted IL along with the regenerated solid material from *Miscanthus* pretreated at 1.0% (w/w) cellulose loading at 140 °C for 18 h. The enzyme reaction proceeded for 16 h, and cellulose conversion was determined with a glucose analyzer. ( ) Mmim DMP pH 4.8; (o) Emim acetate pH 4.8. Lines are provided to guide the eye.
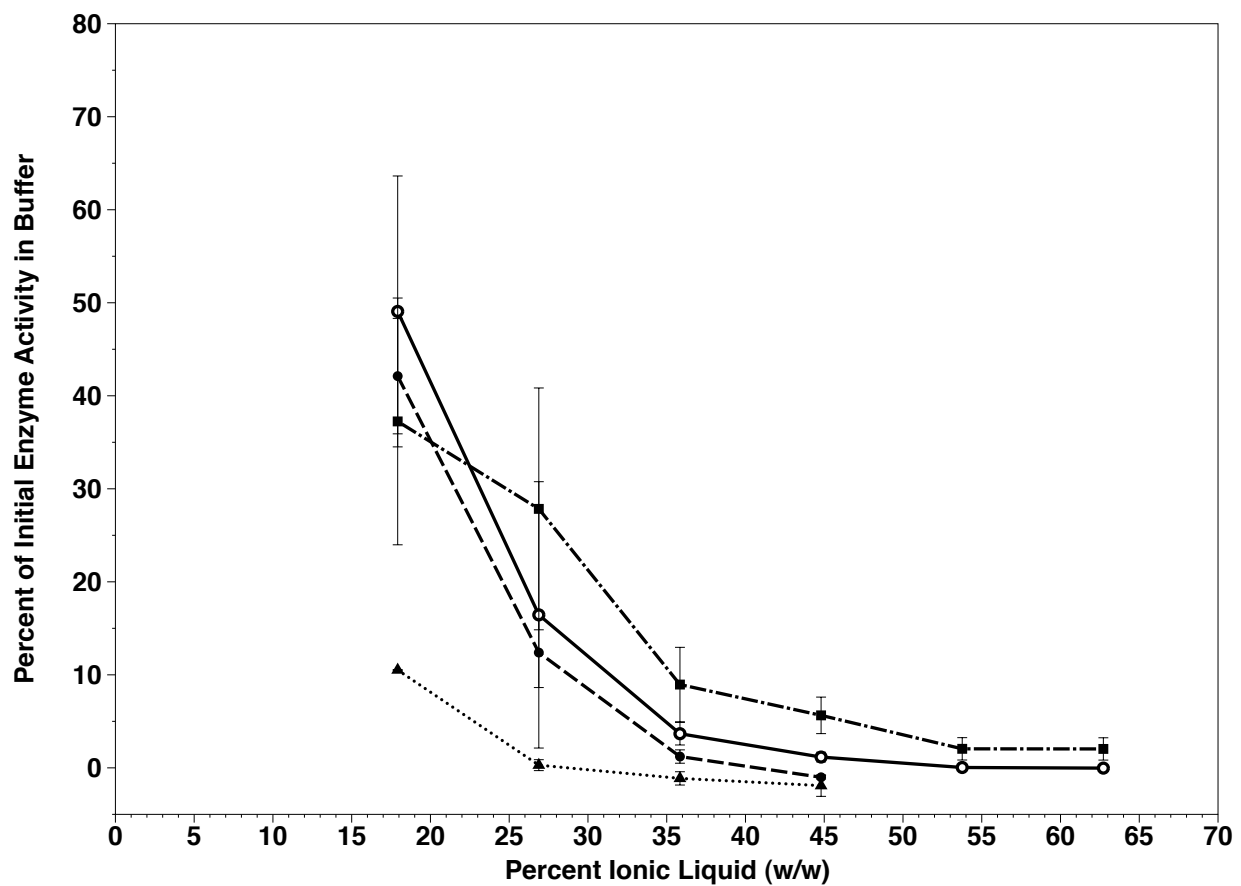
Fig. 7. Subtilisin activity was assayed in the ionic liquid aqueous mixtures above at various concentrations at 25 °C against the substrate (N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide). Product was measured by the absorbance at 410 nm.

% Emim Ac (w/w)	pH	% Mmim DMP (w/w)	pH
10	6.3	10	3.2
19	6.4	20	3.3
27	6.7	30	3.6
35	7.1	40	3.9
		50	4.4

Table 1. Room temperature pH of aqueous solutions of Emim acetate and Mmim DMP.

References:

1. R. P. Swatloski, S. K. Spear, J. D. Holbrey and R. D. Rogers, *J Am Chem Soc*, 2002, **124**, 4974-4975.
2. I. Kilpeläinen, H. Xie, A. King, M. Granstrom, S. Heikkinen and D. S. Argyropoulos, *Journal of Agricultural and Food Chemistry*, 2007, **55**, 9142-9148.
3. S. H. Lee, T. V. Doherty, R. J. Linhardt and J. S. Dordick, *Biotechnol Bioeng*, 2009, **102**, 1368-1376.
4. A. P. Dadi, C. A. Schall and S. Varanasi, *Appl Biochem Biotechnol*, 2007, **137-140**, 407-421.
5. F. van Rantwijk and R. A. Sheldon, *Chemical Reviews*, 2007, **107**, 2757-2785.
6. N. Kaftzik, S. Neumann, M. Kula and U. Kragl, in *Ionic Liquids as Green Solvents: Progress and Prospects*, eds. R. D. Rogers and K. R. Seddon, ACS Symposium Series, 2003, vol. 856, p. 206.
7. M. B. Turner, S. K. Spear, J. G. Huddleston, J. D. Holbrey and R. D. Rogers, *Green Chem*, 2003, **5**, 443-447.
8. N. Kamiya, Y. Matsushita, M. Hanaki, K. Nakashima, M. Narita, M. Goto and H. Takahashi, *Biotechnol Lett*, 2008, **30**, 1037-1040.
9. T. Jeoh, C. I. Ishizawa, M. F. Davis, M. E. Himmel, W. S. Adney and D. K. Johnson, *Biotechnol Bioeng*, 2007, **98**, 112-122.
10. D. Constantinescu, H. Weingartner and C. Herrmann, *Angew Chem Int Edit*, 2007, **46**, 8887-8889.
11. H. V. H. Peter and K.-Y. Wong, *Science*, 1964, **145**, 577-580.
12. G. S. Waldo, B. M. Standish, J. Berendzen and T. C. Terwilliger, *Nat Biotechnol*, 1999, **17**, 691-695.
13. W. W. Ward and S. H. Bokman, *Biochemistry*, 1982, **21**, 4535-4540.
14. W. T. Heller, H. M. O'Neill, Q. Zhang and G. A. Baker, *The Journal of Physical Chemistry B*, 2010, **114**, 13866-13871.
15. Q. Li, X. L. Jiang, Y. C. He, L. Z. Li, M. Xian and J. M. Yang, *Appl Microbiol Biot*, 2010, **87**, 117-126.
16. G. H. Patterson, S. M. Knobel, W. D. Sharif, S. R. Kain and D. W. Piston, *Biophys J*, 1997, **73**, 2782-2790.
17. A. A. Heikal, S. T. Hess and W. W. Webb, *Chem Phys*, 2001, **274**, 37-55.
18. K. M. Alkaabi, A. Yafea and S. S. Ashraf, *Appl Biochem Biotechnol*, 2005, **126**, 149-156.

Chapter 2: Utilization of cellulases from extremophilic organisms for activity in aqueous ionic liquids

Abstract: We had hypothesized that cellulases from thermophilic organisms with high optimal temperatures would be able to function at higher ionic liquid concentrations than cellulases with lower temperature optima. We found this correlation between thermostability and IL-tolerance to hold with most of the enzymes tested. We observed some activity in 70% Mmim DMP (w/w), and we observed a drop in temperature optimum

<u>Assay of thermophilic cellulase activity</u>:

In order to further compare enzyme activity in ionic liquids with activity at high temperatures, the cellulases in Table 1 were assayed in Mmim DMP for activity toward Avicel that had been pretreated with Emim acetate as described in the Methods in chapter 3. Enzymes were prepared by cell-free synthesis. The results are shown in Figures 1 and 2. Two enzymes with high optimal temperatures were observed to be active in high concentrations of Mmim DMP in IL-water mixtures, namely Cel12A from *Pyrococcus furiosus* and Cel5A from *Pyrococcus horikoshii.* Other enzymes in Table 1 were inactive. The activity of *P. furiosus* Cel12A toward Avicel in 49% (w/w) Mmim DMP was the same as in the absence of ionic liquid. At low concentrations of Mmim DMP, the amount of glucose released after 16 hours of enzymatic hydrolysis was higher than in the absence of Mmim DMP. This effect was also observed with the haloalkaliphilic CBHI from *Halorhabdus utahensis,*[1] which exhibited slightly higher activities in 20% (w/w) Emim Cl than in the absence of ionic liquid.

 A similar effect has also been observed in the hyperthermostable glutamate dehydrogenase with a T_{opt} of ~90 ºC from *Pyrobaculum islandicum.*[2] Addition of low or moderate amounts of guanidine hydrochloride or urea, respectively, resulted in an increase in the enzyme's activity at 50 ºC.[2]

Additionally, we observed activity of *Pyrococcus furiosus* Cel12A in concentrations of Mmim DMP as high as 70% (See Figures 3 and 4).   In 70% Mmim DMP, the hydrolysis of IL-pretreated Avicel is approximately 8% of that in buffer at 40 °C, which is the optimal temperature for the enzyme in 70% Mmim DMP. This downward shift in the $T_{opt.}$ with increasing ionic liquid concentration is consistent with the findings of Graham and colleagues when testing their hypothermophilic cellululase in 50% Mmim DMP.[3]

Cell-free protein synthesis

The cellulases shown in Table 2 were produced via cell free synthesis from *E. coli* BL21 Star cell lysate, which had been previously induced to express the chaperones GroEL and GroES.[4] Template DNA had been generated via oligonucleotide synthesis from Genscript with *E. coli* codon usage and cloned into vector pET 302 or pIVEX 2.3 from *E. coli* Top10 and was amplified via maxyprep (Qiagen). Cell free synthesis was carried out as described in Kim, et. al.[4] IPTG (1 mM) was added during cell free synthesis, as pET302 has a lac operator site on the vector.
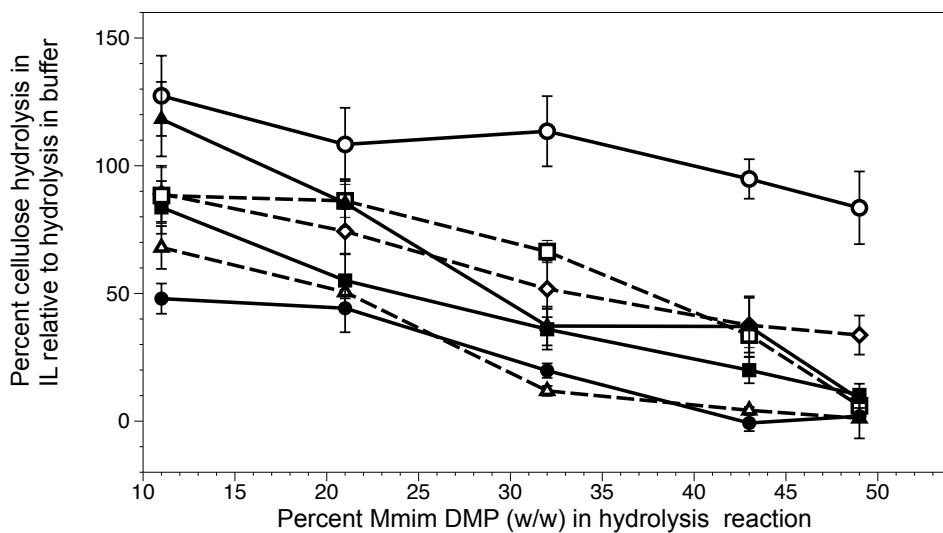
Figure 1: Hydrolysis of 2.5 g/L IL-Avicel in varying concentrations of Mmim DMP at 50 ºC after 16 h of reaction relative to hydrolysis in no IL. Open circles are *P. furiosus* Cel12A. Open squares are *A. cellulolyticus* Cel5A. Closed triangles are *S. lividans* Cel 12A. Open diamonds are *P. horikoshii* Cel5A. Closed squares are *P. abyssi* Cel5A. Open triangles are *B. fibriosolvens* Cel5A. Closed circles are *T. reesei* EGI. The pH was 5.6 except for *A. cellulolyticus* Cel5A and *T. reesei* EGI, which were 4.8.
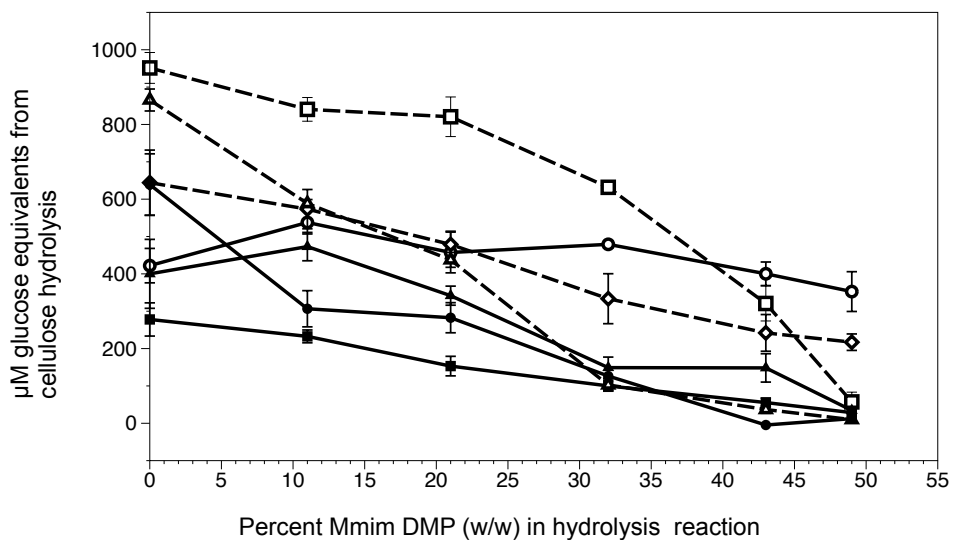
Figure 2: Hydrolysis of 2.5 g/L IL-Avicel in varying concentrations
of Mmim DMP at 50 ºC after 16 h of reaction. Open circles are *P. furiosus*
Cel12A. Open squares are *A. cellulolyticus* Cel5A. Closed triangles are *S. lividans*
Cel 12A. Open diamonds are *P. horikoshii* Cel5A. Closed squares are *P. abyssi*
Cel5A. Open triangles are *B. fibriosolvens* Cel5A. Closed circles are *T. reesei* EGI.
The pH was 5.6 except for *A. cellulolyticus* Cel5A and *T. reesei* EGI, which were 4.8.
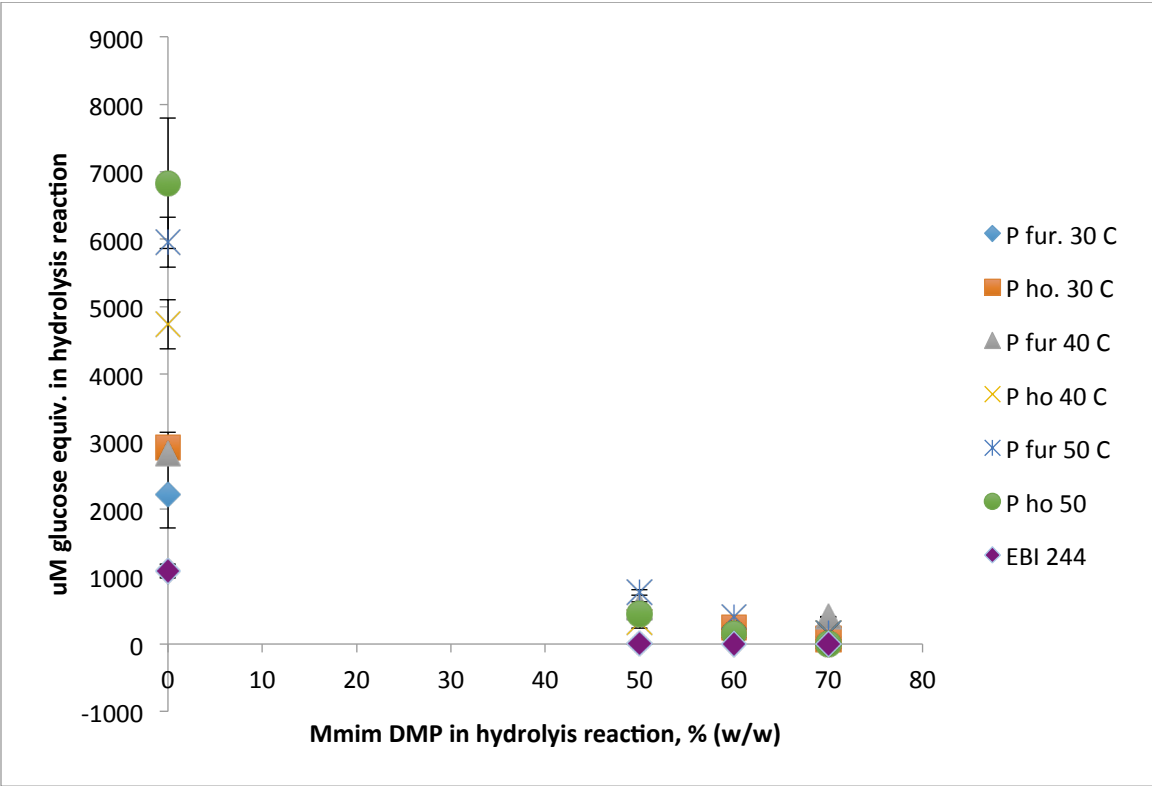
Figure 3: Assay of various cellulases (endoglucanases) from thermophiles in citrate buffer pH4.8 or in varying concentration of Mmim DMP pH 4.8 with varying temperature against Emim acetate-pretreated Avicel after 16 h of hydrolysis time.
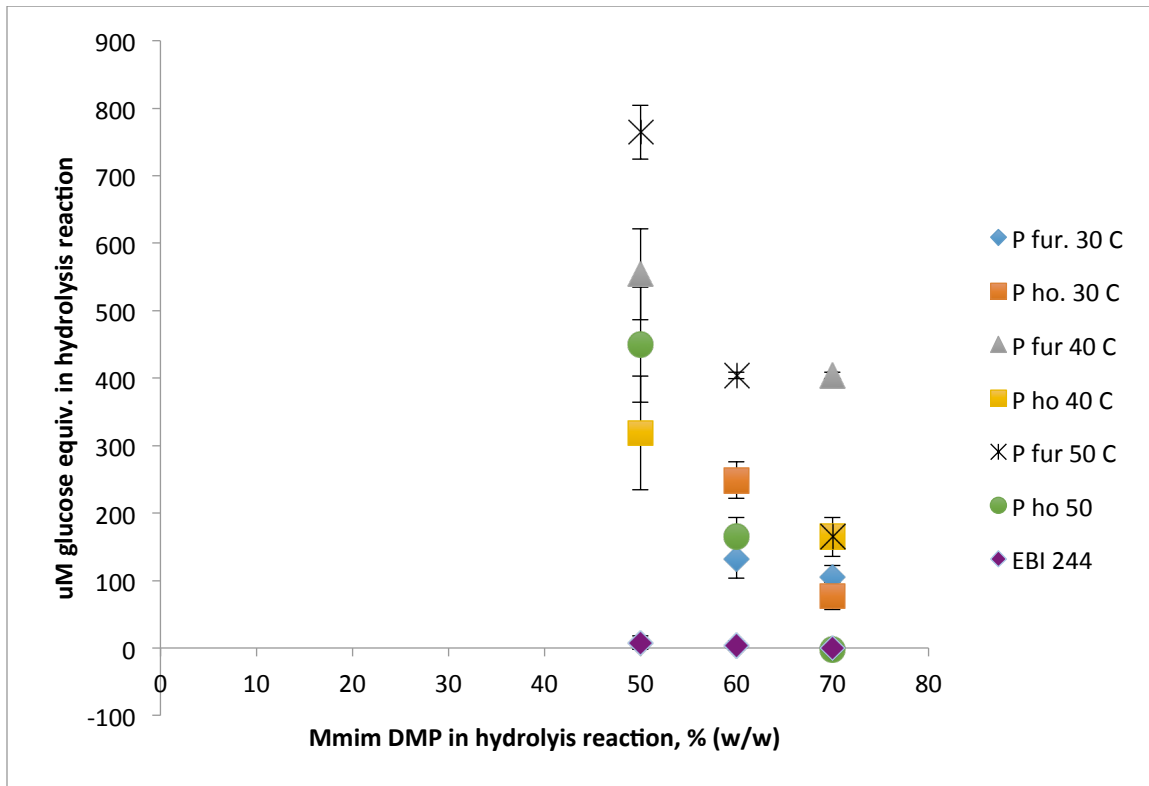
Figure 4. Assay of various cellulases (endoglucanases) from thermophiles in citrate buffer pH4.8 or in varying concentration of Mmim DMP pH 4.8 with varying temperature against Emim acetate-pretreated Avicel after 16 h of hydrolysis time. These data are included in Fig. 3, but this graph highlights the activity in aqueous ionic liquid (Mmim DMP).

References:

1. T. Zhang, S. Datta, J. Eichler, N. Ivanova, S. D. Axen, C. A. Kerfeld, F. Chen, N. Kyrpides, P. Hugenholtz, J.-F. Cheng, K. L. Sale, B. Simmons and E. Rubin, *Green Chem*, 2011, **13**, 2083-2090.
2. C. Kujo and T. Ohshima, *Appl Environ Microbiol*, 1998, **64**, 2152-2157.
3. J. E. Graham, M. E. Clark, D. C. Nadler, S. Huffer, H. A. Chokhawala, S. E. Rowland, H. W. Blanch, D. S. Clark and F. T. Robb, *Nat Commun*, 2011, **2**, 375.
4. T.-W. Kim, H. A. Chokhawala, D. Nadler, H. W. Blanch and D. S. Clark, *Biotechnology and Bioengineering*, 2010, **107**, 601-611.

Chapter 3

Engineering ionic liquid tolerant cellulases for biofuels production

Abstract: Lignocellulosic biomass dissolution in ionic liquids can provide a very effective pretreatment prior to enzymatic saccharification of cellulose for biofuels production. For the purpose of combined pretreatment and enzymatic hydrolysis, we evolved CBHI enzyme variants of *Talaromyces emersonii* Cel7A, via DNA shuffling, to be more active and stable than wild type *T. emersonii* Cel7A or *Trichoderma reesei* Cel7A in aqueous-ionic liquid solutions (up to 43% (w/w) 1,3-dimethylimdazolium dimethylphosphate and 20% (w/w) 1-ethyl-3-methylimidazolium acetate). These enzyme variants were also more active and stable at elevated temperature than the wild type enzymes. We also demonstrated that thermophilic cellulases from thermophilic organisms are active in higher ionic liquid concentrations than mesophilic cellulases.

Introduction:

In 2002, Rogers and colleagues identified the room temperature ionic liquid, 1-butyl-3-methylimidazolium (Bmim) chloride, to be an excellent solvent for cellulose.[1] This ionic liquid (IL) was shown to be able to dissolve up to 10% (w/w) cellulose. When microwaved, up to 25 % (w/w) cellulose could be dissolved. Subsequently, Dadi and colleagues[2] reported that cellulose precipitated from certain ionic liquids by addition of an antisolvent such as water could be readily hydrolyzed by cellulase enzymes. Turner and colleagues, however, reported hydrolysis of precipitated cellulose in the presence of the ionic liquid 1-butyl-3-methylimidazolium (Bmim) chloride to be difficult, due to denaturation of the cellulases.[3]

Lee et al. identified 1-ethyl-3-methylimidazolium (Emim) acetate as able to dissolve wood and separate the lignin from the holocellulose.[4] Ethoxylated ionic liquids with acetate anions[5] were shown to permit enzymatic hydrolysis of the cellulosic content of lignocellulosic biomass in the presence of the ionic liquid. Kamiya et al. identified Emim diethylphosphate as able to support cellulase activity at ionic liquid concentrations as high as 40 % (v/v).[6] Combining lignocellulose pretreatment by dissolution in ionic liquids with enzymatic hydrolysis in the same vessel offers potential increases in process efficiency.

We previously examined protein stability in a variety of other ionic liquids using green fluorescent protein as a model for assessing cellulase stability.[7] *Trichoderma reesei* cellulases were shown to be active in 40% (w/w) 1,3-dimethylimidazolium dimethylphosphate (Mmim DMP).[7]. We also showed β-glucosidase to be stable in up to 60% (w/w) solutions of Mmim DMP in water. Mmim DMP was thus selected as the model solvent to examine the activity of cellulase variants in the present work.

Cellulases from *Trichoderma reesei* are comprised of endoglucanses and exoglucanases (cellobiohydrolases). Endoglucanases break the glycosidic bonds within a polyglucan chain, while cellobiohydrolases cut from the chain ends.[8] CBHI comprises approximately 60% of the *T. reesei* cellulase mixture, and thus was chosen for the present study.[9]

Directed evolution has been shown useful in improving enzyme kinetics and substrate specificity, in addition to solvent and/or thermal tolerance. Brayan and coworkers mutagenized subtilisin in a random fashion and screened for mutants for activity at temperatures higher than the wild type optimal temperature, finding an increase in the optimal temperature ($T_{opt.}$) of 2.4°C in at least one mutant.[10] Chen and Arnold used directed evolution to engineer subtilisin for increased catalytic activity in dimethylformamide using multiple rounds of error prone PCR and screening.[11]

Another approach to directed evolution is DNA shuffling, which was employed in the present work. DNA shuffling was first described by Stemmer in 1994 to evolve β-lactamase for hydrolysis of the antibiotic cefoxime.[12] DNA shuffling involves digesting homologous genes and allowing the DNA pieces to align at homologous regions and then extend them via PCR, resulting in chimeric mutant genes.[12] Directed evolution has been applied to cellulases in efforts to increase their activity and/or optimal temperature. These methods have employed error-prone PCR and DNA shuffling.[13] In the present work, we used a biased clique method for DNA shuffling because this method had been predicted to produce a more active library (Moore and colleagues[14]) and demonstrated by Dana, et al.[15]. Previous efforts to improve CBHI from *Talaromyces emersonii* used structure-guided site-directed mutagenesis.[16-18] In contrast, biased clique shuffling does not require this structural information.

Thermophilic cellulases, including several thermophilic and IL-tolerant cellulases,[19] show activity at higher ionic liquid concentrations than their mesophilic counterparts.[20] One

cellulase, reported by Graham and colleagues, has a T_{opt} of 109°C and was shown to be active in 50 % (v/v) 1,3-dimethylimidazolium dimethylphosphate / water solutions.[21] The presence of negatively charged residues on the protein surface has been postulated to be important for IL-tolerance, as evidenced by a haloalkaliphilic cellulase that has been reported to be stable in aqueous-IL solutions.[22] The present work further explores the relationship between thermotolerance and the ability of cellulases to function in certain IL-water solutions.

Results and Discussion:

Screening and initial analysis of variants

CBHI variants were selected and purified for further study after screening the activity of each variant toward Emim acetate-pretreated Avicel (see Methods) in aqueous solutions containing 20% (w/w) Mmim DMP, at pH 4.8 and in citrate buffer. The variants were generated via biased clique shuffling (Methods) from the parent *Talaromyces emersonii* (Te) Cel7A. The variants that were selected all released more sugars from the pretreated Avicel in the presence of 20% (w/w) Mmim DMP than the variant "2I13," described by Dana and colleagues.[15] The variant 2I13 had a $T_{opt.}$ of ~60°C and exhibited the highest residual activity after incubation at 65 °C for 24 h of the mutants studied.[15] Two variants (1M10 and 2K15) were also shown to exhibit activity toward 4-methylumbelliferyl lactoside (MU-lac) at 70 °C for at least 6 days.[23]

Of the variants selected, (see "Selection of enzyme variants" in Materials and Methods) 12 were expressed for further analysis. After purification, they were assayed for their activity toward Emim acetate-pretreated Avicel in 43% (w/w) Mmim DMP (Figure 1; activity in other concentrations of Mmim DMP is shown in S1a, and S1b). Also tested was *T. reesei* Cel7A, purified from Celluclast. In 43% (w/w) Mmim DMP, two variants, 2K15 and 1M10, produced more glucose than any of the variant or wild type enzymes tested, including 2I13, 2E10, and 1G21, the stable variants identified by Dana, et. al.[15] *T. reesei* Cel7A was essentially inactive under this condition.

Te Cel7A (expressed in *S. cerevisiae*) and *T. reesei* Cel7A (purified from Celluclast) and variants 1M10, 2K15, 2I13, and 1G21 were assayed in Emim acetate/water solutions under the same conditions as those employed with Mmim DMP. Figure 2 shows the glucose released from the hydrolysis of Emim acetate-pretreated Avicel in 20% (w/w) Emim acetate relative to glucose released from hydrolysis in buffer. The results are similar to those in 43% (w/w) Mmim DMP, with 1M10 and 2K15 giving the highest cellulose hydrolysis in 20% (w/w) Emim acetate relative to buffer. Figure S2a and S2b are similar to S1a and S1b, although hydrolysis is only shown in Emim acetate at up to 30% (w/w) concentration because Mmim DMP is more supportive of cellulolytic activity.[7]

In no or in low Mmim DMP or Emim acetate concentrations, we observed noticeably more glucose released by *T. reesei* Cel7A than from the other enzymes. This effect is likely due to the glycosylation pattern from the *T. reesei* expression compared to that resulting from *S. cerevisiae* expression (Chokhawala, personal communication).[24] This effect was also apparent with expression in the filamentous fungus, *Neurospora crassa.* We expressed some of these mutants and wild type Te Cel7A in *Neurospora crassa* and assayed them for stability after pre-incubation as described in the next section. We observed the *N. crassa*-expressed enzymes all to be more stable than those expressed in yeast (data not shown).

Enzyme stability in ionic liquid/water solutions and at elevated temperature

We examined the stability of selected CBHI variants in aqueous-ionic liquid solutions by incubation in 43% (w/w) Mmim DMP or 20% (w/w) Emim acetate at 50 °C. Residual activity at 50 °C was measured after pre-incubation in the aqueous- IL solution, followed by 10 fold dilution into a buffered solution containing the substrate MU-lac. Figures 3 and 4 show the results for *T. reesei* Cel7A, Te Cel7A, 1M10, and 2K15 and the following mutants identified by Dana, et. al.[15]: 2I13, 2E10, and 1G21. We see that Cel7A from *T. reesei* and Cel7A from *T. emersonii* (expressed in *S. cerevisiae*) were the least stable of the enzymes assayed.

We observed the 1M10 variant to be stable even after 24 h of incubation in 20% (w/w) Emim acetate. 1M10 and 2K15 had the highest residual activity after incubations of 1 h or less in 43% (w/w) Mmim DMP, and also exhibited hydrolysis in 43% (w/w) Mmim DMP of Avicel pretreated with Emim acetate (Figure 1). Since 2I13 lost activity after incubation in 43% Mmim DMP very rapidly, its residual activity may result from reversible inactivation in 43% Mmim DMP. The wild type enzymes were the least stable after incubation in either of the IL conditions described above, becoming irreversibly inactivated in 43% Mmim DMP.

The thermostability of selected variants was examined at 65 °C, and is shown in Figure 5. It was observed that the three enzymes (*T. reesei* Cel7A, Te Cel7A, and the variant 2E10) that quickly lost activity after incubation in 43% (w/w) Mmim DMP at 50 °C also lost activity irreversibly after incubation at 65 °C. A relationship between the thermostability of the mutants and their stability in aqueous-ionic liquid solutions was observed. Enzymes that were stable in 20% Emim acetate at 50 °C were also stable in buffer at 65°C (Figure 6), with 1M10 being the most stable at each condition.

It was later determined that the Te enzyme used for some of these assays had included and additional alanine insertion at the N-terminus. When this removed, there was no significant difference between the stability of these two variants. The figures showing relative hydrolysis amounts reflect the enzyme without the alanine, while, for internal consistency, the figures (except those showing glutamine cyclase-treated enzymes) showing absolute glucose concentrations have the enzyme with the alanine insertion.

Structural basis of stability

Figure 7 shows a model constructed with Visual Molecular Dynamics[25] based on the crystal structure[26] of Te Cel7A. The mutations present in 2K15 are highlighted in yellow and are listed in Table 3, while the mutations for 1M10 are in Table 4. The mutations for 2K15 and the three previously studied by Dana, et al.[15] are on the surface of the protein. The only mutations shared by 2K15 and 2I13 are at sites 58 and 60. The same mutations are present in both variants, P58T and Y60L. These are in a loop or turn region in the structure. It has been shown previously that proteins with smaller loops and more compact structures are correlated with more thermostable proteins.[27, 28]

To observe enzyme conformation in the presence of ionic liquids we performed differential scanning calorimetry of several CBHI variants in acetate buffer pH 5.0 and in aqueous Mmim DMP. The melting temperatures are shown in Table 2. These data demonstrate that the loss of enzymatic activity in the ionic liquid is due to global unfolding of the enzyme. For example, the wild type Te enzyme (without glutamine cyclase treatment) has a Tm in 43% Mmim DMP (without incubation in this condition for any significant time) of 46.0 °C, which is below the assay temperature of 50 °C. The 1M10 variant has a Tm of 50.1 °C, which may indicate why some activity is observed with 1M10 in 43% Mmim DMP. Reversal of unfolding was not observed via DSC in 43% Mmim DMP.

Upon transformation into and expression in *Neurospora crassa* of the wild type Te enzyme into by Christy Roche, we observed the enzyme in this system to be more stable than the same enzyme expressed in *S.* cervesiae (data not shown). Upon looking at the PDB structure of the *T. emersonii* CBHI, a PCA[26] residue was observed. Craig Dana then initially tested to see if this modification was due to glutamine cyclase, and it was verified by DSC.[29]

Thus (in order to make these enzymes as close to those expressed in a filamentous fungus as possible), we proceeded to produce for this study, the 1M10, 2I13 variants, and wild type Te enzyme with no His tag and then, after purification subject them to glutamine cyclase treatment (see Methods).

Cyclization of the N terminal glutamine increased the melting point of all enzymes substantially (approximately 10 °C). Even after glutamine cyclization of both variants, the 1M10 variant was still more stable than the Te wild type enzyme, having a higher Tm both in buffer alone and in 43% Mmim DMP.

Additionally, we observed two peaks for the DSC trace for the 2I13 variant without exogenous glutamine cyclase treatment. This sample was much older than the others and had been stored at 4 °C in 20 mM acetate buffer pH 5 for 18 months. It appears that some of the sample had undergone N-terminal glutamine cyclization spontaneously, as the second peak has an identical Tm in buffer to that of the 2I13 variant when exogenous glutamine cyclase was added. It is possible that glutamine cyclization for part of the population of enzyme results in the plateau of cellulase activity after various periods of incubation in ionic liquid or at elevated temperature.

We repeated most of the previous assays using the QC-treated enzyme and Te without QC treatment as a comparison. Figures 8-11 and S3a-S4b show this. What we observe with these assays is that after QC treatment all three enzyme variants function with similar activity in aqueous Mmim DMP and Emim acetate against Avicel and with similar residual activity against 4-MU-lactoside after prior incubation in 43% Mmim DMP at 50 °C and in citrate buffer pH 4.8 at 65°C. In fact the QC treated enzymes retain almost all of their activity after 24 h of incubation at 65 °C, whereas Te without QC treatment had lost all of its activity by then.

Conclusions:

Using directed evolution via DNA shuffling, a variant cellulase was isolated that was more active and stable in aqueous-ionic liquid solutions and at elevated temperatures than the wild type enzyme. Cellulases with higher optimal temperatures were active at higher concentrations of ionic liquid in ionic liquid/water mixtures. However, we see the importance of screening in native-like conditions, and believe future evolution studies in the same system should include addition an active glutamine cyclase either produced by the expression host or added exogenously.

Materials and Methods:

Mutant Library Construction:

DNA shuffling

The library of chimeric CBHI genes (including catalytic domain, linker, and carbohydrate binding module) was generated via DNA shuffling as described in Dana, et. al.[15] Briefly, 11 Cel7A (CBHI) genes were selected based on homology to Cel7A from *Taleromyces emersonii*. The genes selected were from the organisms listed in table 1. They were synthesized via oligonucleotide synthesis from Genscript for *T. emersonii* Cel7A and from DNA 2.0 for the others. The codons were optimized for expression in *S. cerevisiae* using the companies' propriety algorithms. The genes were cloned into pCu424 or pCu425 and were amplified via polymerase chain reaction (PCR).

Each library was generated using the methods in Dana, et. al.[15] Briefly, the library DNA was digested using DNaseI. Digestion was quenched with EDTA. The library was then reassembled using using PCR and gel purified. After cloning into pCu424 and amplification of the library in *E. coli* Top10, the library was transformed in *S. cervesiae* YVH10, which overexpresses protein disulfide isomerase and was provided by the Wittrup group.[30] The PMR1 gene had also been disrupted.[15] Transformants were selected by plating onto DOBA/SC-Trp (both from Sunrise Science Products, using their recommendations) with 100 mg/L adenine hemisulfate. The first library from a DNA pool of which 50% was the *T. emersonii* Cel7A gene and 5% was each of the other genes from the organisms in Table 1.

Dana and colleagues identified three thermostable mutants using that library.[15] The library used in this study was then generated from a pool of which 25% was the *T. emersonii* Cel7A gene. Each of the three thermostable mutants composed 8.33% of the library's DNA pool, while the 10 genes from the organisms in Table 1. (other than *T. emersonii*) composed 5% of the library. This library was cloned, transformed, and selected as for the first library.

Chimeric cellulase production:

From the selection plates, colonies were picked using a Genetix Qpix2 colony picker and grown in 250 μl of YPD media (50 g/L) with 100 mg/L of Adenine hemisulfate (YPAD) in 96 well plates. After 3 days of shaking at 30 °C at 250 rpm, the supernatants were pipetted off and replaced with YPAG with 500 μM $CuSO_4$ (for heterologous protein induction). The YPAG media contains 10 g/L yeast extract, 20 g/L peptone, and 20 g/L galactose (galactose replaced the dextrose to minimize any glucose background in subsequent cellulase activity assays). Of this suspension, 10 μl were resuspended in YPAG with 15% glycerol. After 3 days of growth in the induction media at 25 °C, the plates containing the heterologously expressed protein in the supernatant were frozen at -20 °C prior to assay. The glycerol stocks were then stored at -80 °C.

Library Screening:

Substrate preparation:

The chimeric proteins were assayed for hydrolysis of 2.5 g/L IL-pretreated Avicel. The substrate was prepared by dissolution of 1 g of Avicel into 10 g 1-ethyl-3-methylimidazolium acetate (Iolitec), which was incubated for 1 h at 100 °C with stirring to produce an optically clear solution. The Avicel was then regenerated from the IL by cooling the IL to 70 °C and adding 10 ml of water that was also at 70 °C. The resulting material was centrifuged at 3220 g for 5

minutes, and the supernatant was removed. The wash/centrifuge step was repeated 4 times over, and then the IL-pretreated Avicel was diluted to 1.25% in 50 mM citrate buffer pH 4.8.

Assay set up:

In 384 well plates (Corning 3958), 14 μl of 1.25% IL-pretreated Avicel was pipetted using 250-μl wide orifice tips (Axygen) with a Biomek FXP robot (Beckman Coulter) from a slurry that was homogenized using a magnetic tumble stirrer (V&P Scientific) during pipetting. 51 μl of 27.5% (w/w) 1,3-dimethylimidazolium dimethylphosphate (Ioltec) pH 4.8 or 50 mM citrate buffer pH 4.8 was then pipetted into the plates. The frozen yeast supernatant containing the expressed protein was thawed to room temperature and then 5 μl of it was pipetted with the Biomek FXP robot. To determine background glucose in the yeast supernatant, 65 μl of 50 mM citrate pH 4.8 was added to 5 μl of yeast supernatant from each mutant in the library. The experimental and background plates were then sealed and incubated at 50 °C with shaking at 150 rpm in a Thermo Scientific MaxQ6000 incubator shaker for 16 h and then frozen before further analysis.

Glucose equivalent analysis.

After thawing the plates, the cellulose hydrolysis reaction was diluted with 50 mM citrate 1:1. Then, after centrifugation of the dilution plates at 1610 g for 2 min, 3 μl of the diluted hydrolysis were mixed with 3 μl of β-glucosidase (5 mg/ml, purified from almonds) and incubated at 37 °C for 2 h. Glucose was then determined by Amplex Red analysis as done by Kim, et. al.[31]

Selection of enzyme variants:

Due to differences in protein expression levels in the library, the variants selected for further study were based on the amount of glucose released in 20% Mmim DMP at 50 °C after 16 h. All selected variants produced more glucose than the 2I13 mutant.[15] Ranking was based on sugar released from IL-pretreated Avicel in the presence of IL divided by the sugar released without the IL present. Selected mutants from this ranking had at least a two-fold signal to background ratio.

Characterization of Selected Enzymes

Protein expression of selected mutants and wild type enzymes:

The enzymes listed in Fig 1 were revived from the frozen glycerol stocks by growth in 5 ml of YPAD until saturation. Then, using 2 ml of the saturated culture, 100 ml cultures of SC-Trp in 300 ml Ultra Yield flasks (Thomson Instrument Company) for each gene were inoculated and allowed to shake at 200 rpm at 30 °C for 3 days. Protein induction was done as for the high throughput library with centrifugation of the cultures and replacing the supernatant with YPAG with 500 μM CuSO4 and then shaking at 200 rpm at 25 °C for 3 days.

Purification of selected mutants:

Each mutant enzyme was collected from the supernatant of the induction culture by centrifugation at 3220 g. The supernatant was concentrated using 10 kDa cutoff 50 ml Sartorius spin concentrators until 90% of the copper had been removed and exchanged with the 150 mM

NaCl, 50 mM Tris, pH 7.6. The samples were then equilibrated to 10 mM imidazole as well and then frozen at -20 °C until purification. Purification was accomplished using an Äkta Purifier with a 5 ml His Trap column (GE Healthcare) with elution with increasing imidazole concentration. Imidazole was then removed via buffer exchange with 20 mM Sodium acetate pH 5.0 via spin concentration.

Enzymes for glutamine cyclase treatment had had their His Tag removed by Craig Dana. These enzymes were purified via anion exchange chromatography. The first column was a HiPrep Q (GE Healthcare) column, and the second was a MonoQ column, also from GE Healthcare. Elution was performed with a in 50 mM Na phosphate pH 7.4 with a gradient of 30-45 min at 4 ml/min of 0-0.5 M NaCl.   Active fractions were identified with activity on 4-MU lactoside (see below).

Assays against IL-pretreated Avicel:

Based on OD280, the mutant proteins were normalized in concentration so that the protein concentration in the hydrolysis assay would be 0.2 μM. IL-pretreated Avicel and aqueous-IL or 50 mM citrate buffer pH 4.8 were added to 96 well assay plates with the Biomek robot. Thermophilic cellulase experiments were conducted at pH 5.6, except for *A. cellulolyticus* Cel5A, which was at pH 4.8. Then, the purified enzymes were added, and the assay plates were then centrifuged for 2 min at 1610 g. The plates were sealed and shaken at 150 rpm at 50 °C for 16 h. They were then analyzed for glucose as for the high throughput assay, using 3 h of incubation time with the β-glucosidase.

Assays against 4-Methylumbelliferyl lactoside (MU-lac):

These assays were conducted in PCR tubes in thermocyclers. Cellulases were incubated in 50 mM citrate pH 4.8 or in aqueous-IL at 0.3 μM protein concentration. Cellulase activity against MU-lac (Carbosynth) was measured as follows: The cellulase was diluted 10 fold (to 0.03 μM enzyme reaction concentration) from the incubation conditions (buffer or aqueous-IL) into 90 μl 930 μM Mu-lac in 50 mM Na acetate pH 5, resulting in reaction concentration of 837 μM MU-lac at 100 μl. The MU-lac hydrolysis was conducted at 50 °C for 10 min. The MU-lac hydrolysis was stopped by addition of 25 μl of 1 M glycine pH 10.2, and the MU-lac hydrolysis was then determined by fluorescence at an excitation of 365nm and an emission of 470 nm.

Differential Scanning Calorimetry:

Using a Nano DSC (TA instruments), we measured melting temperatures of cellulases in the presence of ionic liquid and in buffer alone. The buffer was 50 mM Na acetate pH 5.0. Enzyme concentration was approximately 0.2 mg/ml in the sample. Scans were done at a step rate of 1 °C/min after equilibration and prior degassing of the sample under vacuum for 10 min.

Glutamine cyclase treatment of yeast-expressed Cel7A variants:

Glutamine cyclase (QC) was purchased from Sino Biological. 0.2 mg/ml QC enzyme was diluted ten fold into a cellulase stock that was 80-100 μM Cel7A in a 50 mM Na phosphate pH 7.4. The reaction was then incubated at 30 °C for 2 days.
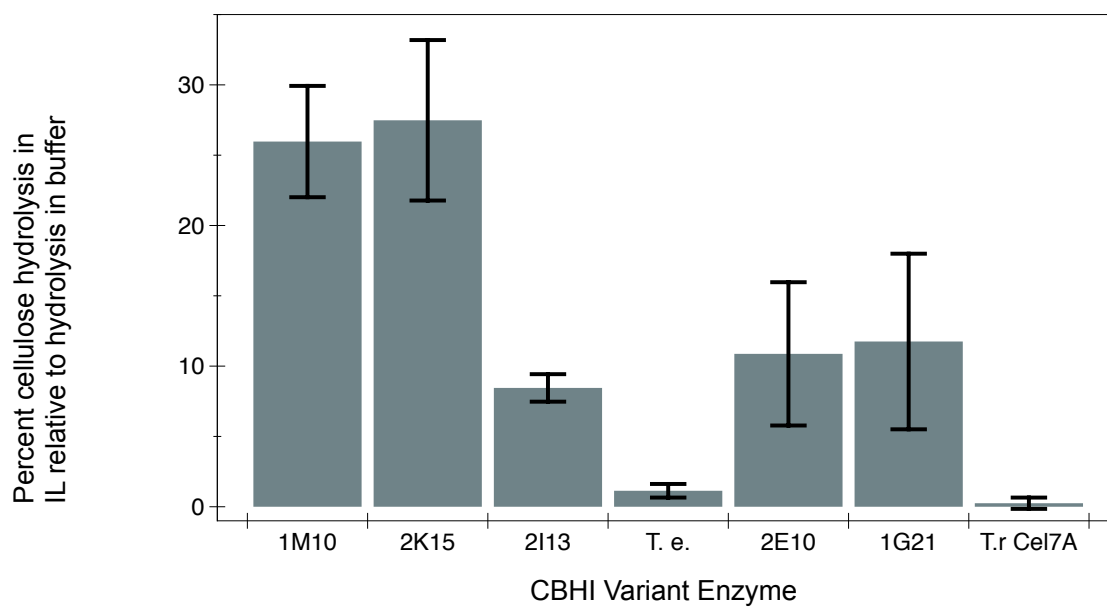
Figure 1. Hydrolysis of 2.5 g/L Emim acetate-pretreated Avicel at 16 h of reaction at 50 °C in 43% Mmim DMP pH 4.8 relative to hydrolysis in citrate buffer pH 4.8, with cellulases at 0.2 μM enzyme loading.
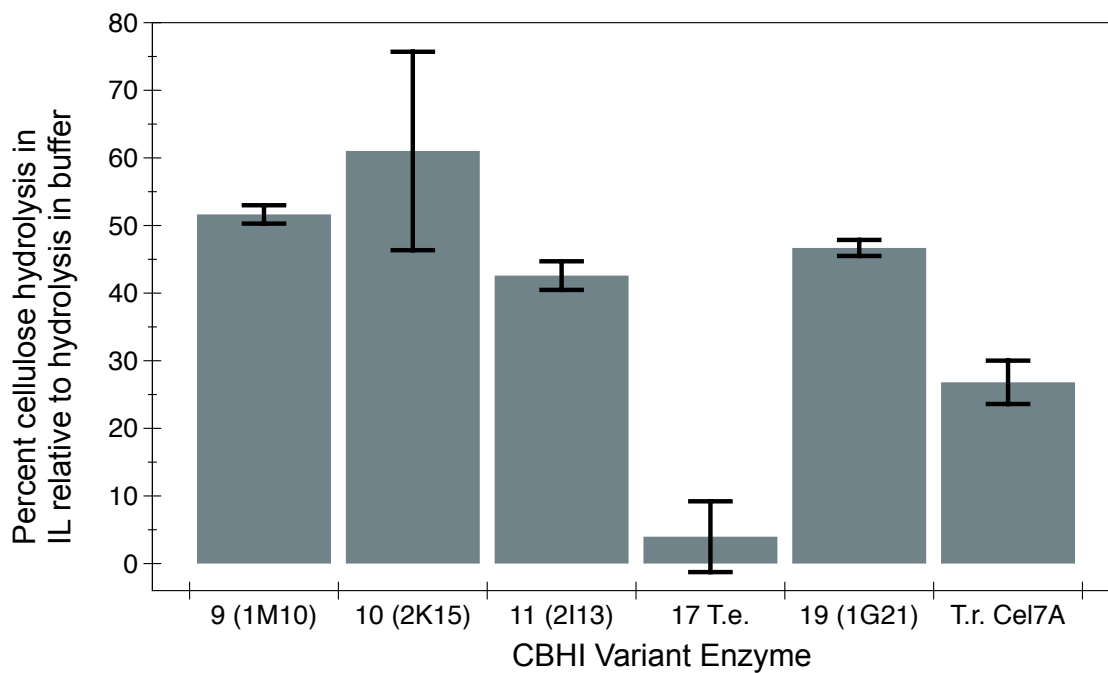
Figure 2. Hydrolysis of 2.5 g/L Emim acetate-pretreated Avicel at 16 h of reaction at 50 °C in 20% Emim acetate pH 4.8 relative to hydrolysis in citrate buffer pH 4.8, with cellulases at 0.2 μM enzyme loading.
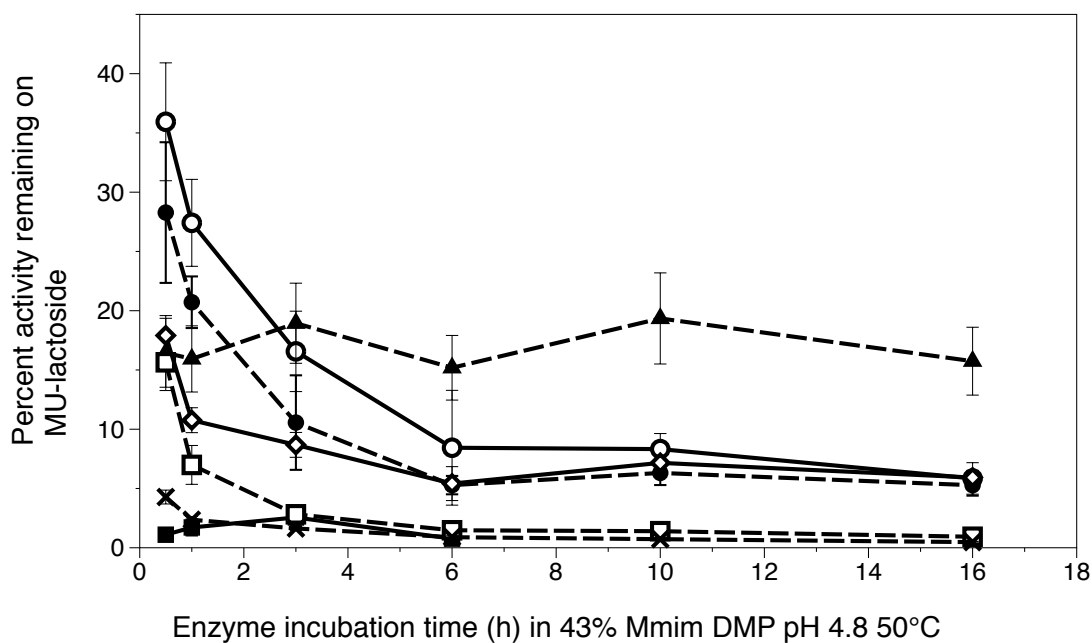
Figure 3. Activity of selected CBHI variants, assayed on MU-lac in 50 mM Sodium acetate at 50 °C after preincubation for varying periods of time in 43% (w/w) Mmim DMP at 50 °C. Closed triangles are 2I13. Open circles are 1M10. Closed circles are 2K15. Open diamonds are 1G21. Open squares are 2E10. Closed squares are T.e. Cel7A. X's are *T. reesei* Cel7A.
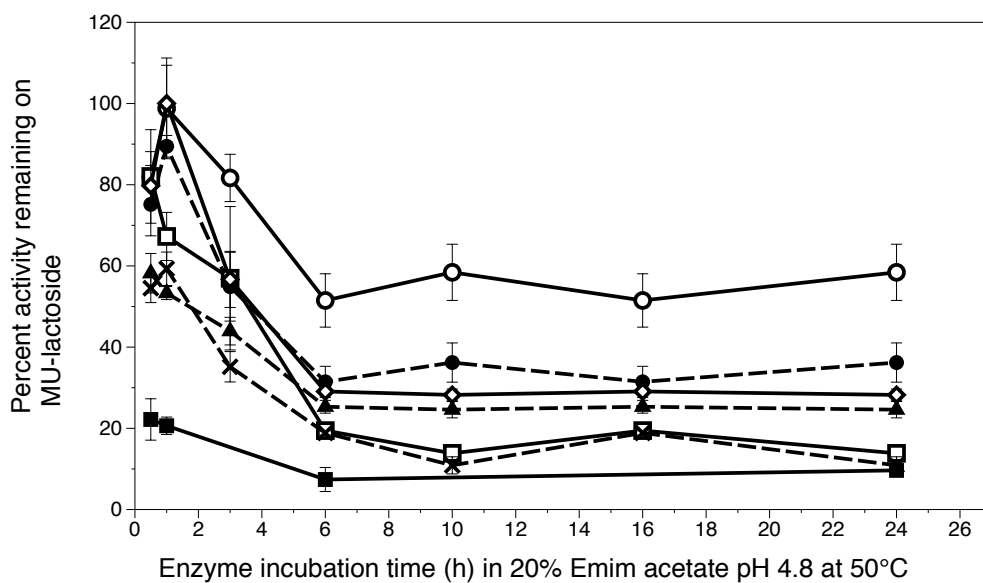
Figure 4.   Activity of selected CBHI variants, assayed on MU-lac in 50 mM Sodium acetate at 50 °C after preincubation for varying periods of time in 43% (w/w) Mmim DMP at 50 °C. Closed triangles are 2I13. Open circles are 1M10. Closed circles are 2K15. Open diamonds are 1G21. Open squares are 2E10. Closed squares are Te Cel7A. X's are *T. reesei* Cel7A.
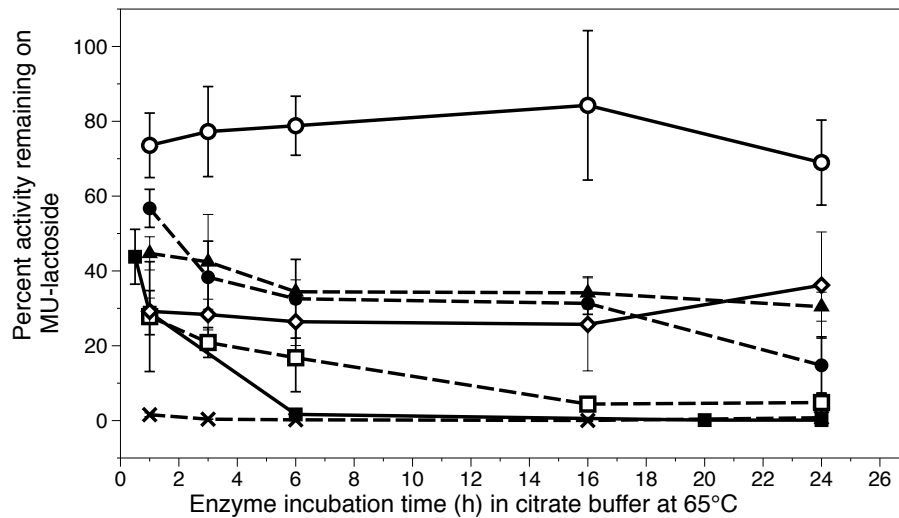
Figure 5. Residual activity of selected CBHI variants at 65 ºC as a function of time. The assay was based on MU-lac at 50 ºC in 50 mM sodium acetate after preincubation for varying periods at 65 ºC. Closed triangles are 2I13. Open circles are 1M10. Closed circles are 2K15. Open diamonds are 1G21. Open squares are 2E10. Closed squares are Te Cel7A. X's are *T. reesei* Cel7A.
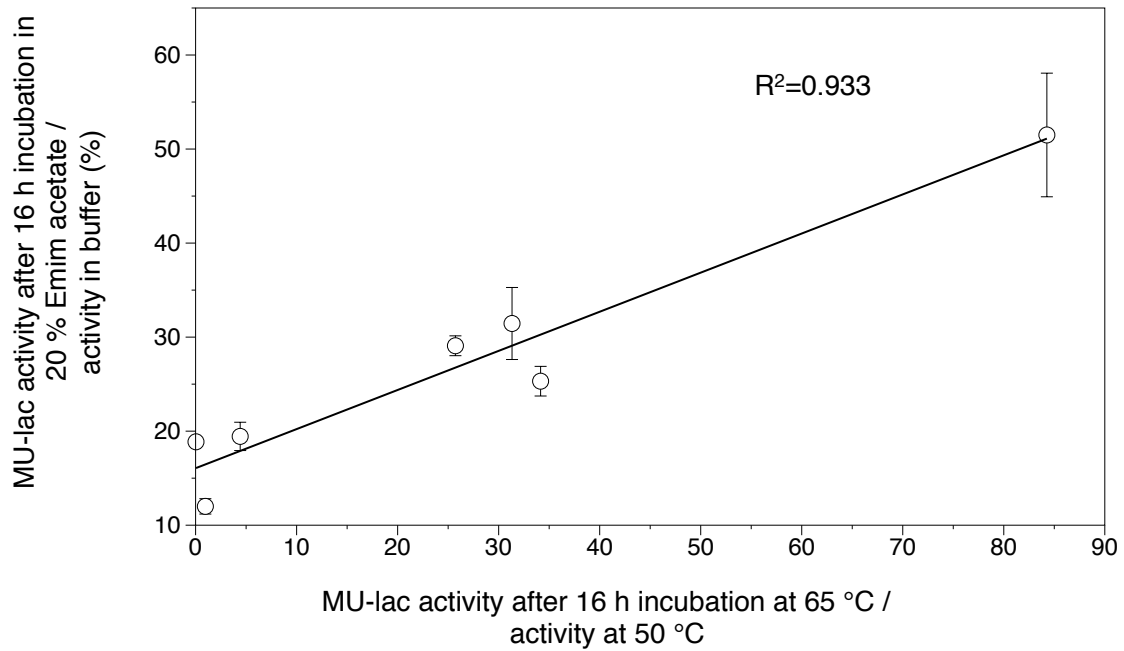
Figure 6: Chimeric or wild type CBHI activity in citrate buffer at 50 °C against MU-lac after prior incubation in buffer at 65 °C or 20% Emim acetate at 50 °C for 16 h. Each point corresponds to a different CBHI enzyme.
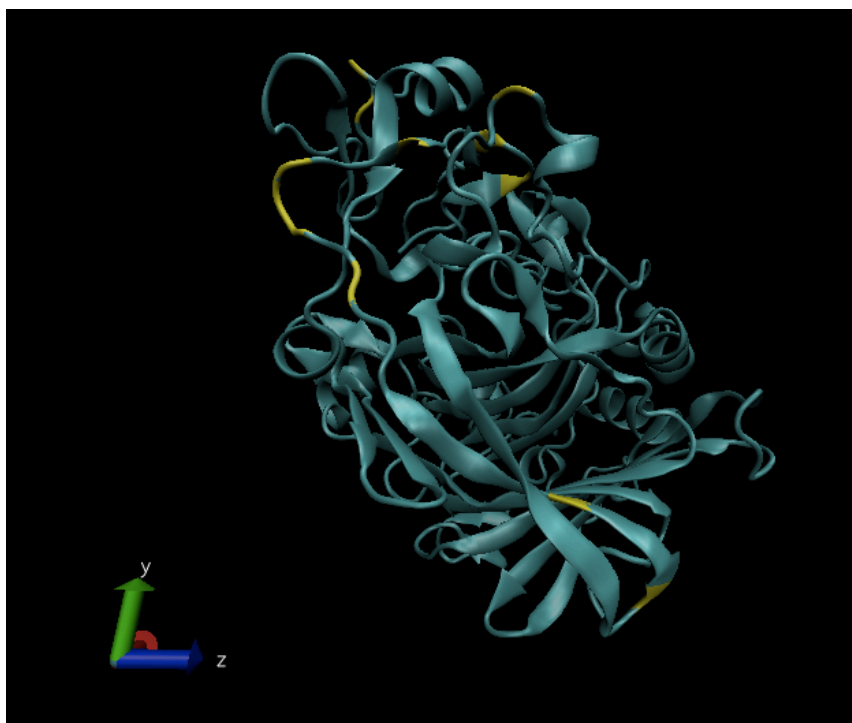
Figure 7. Crystal structure model of *T. emersonii* Cel7A (PDB ID: 1Q9H) generated with Visual Molecular Dynamics.   Mutations in variant 2K15 are highlighted in yellow.
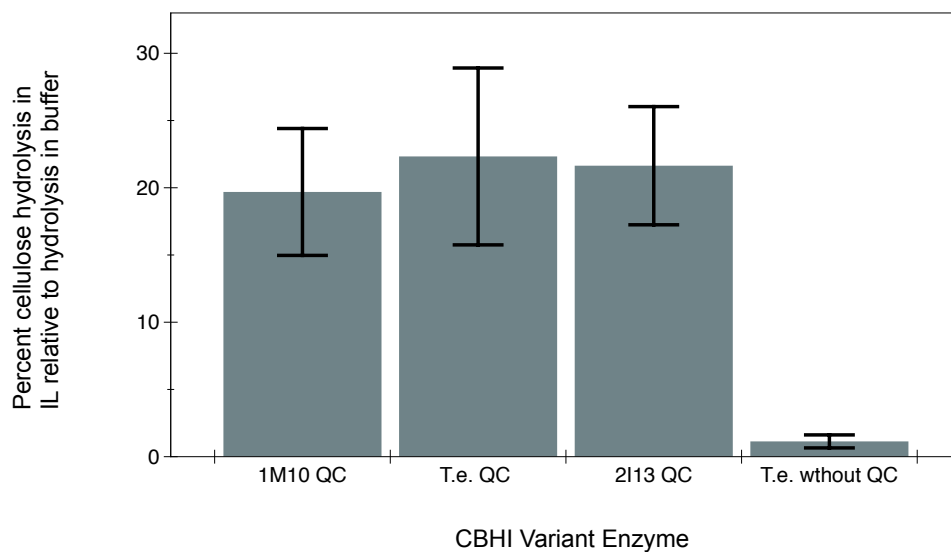
Figure 8. Hydrolysis of 2.5 g/L Emim acetate-pretreated Avicel at 16 h of reaction at 50 °C in 43% Mmim DMP pH 4.8 relative to hydrolysis in citrate buffer pH 4.8, with cellulases at 0.2 μM enzyme loading. In this case the enzymes labeled "QC" were treated with glutamine cyclase as described in Methods.
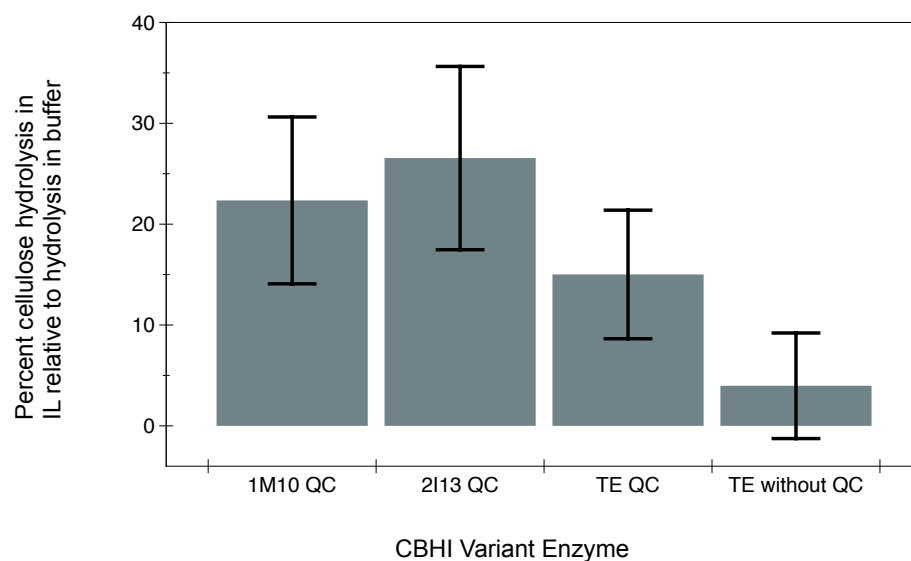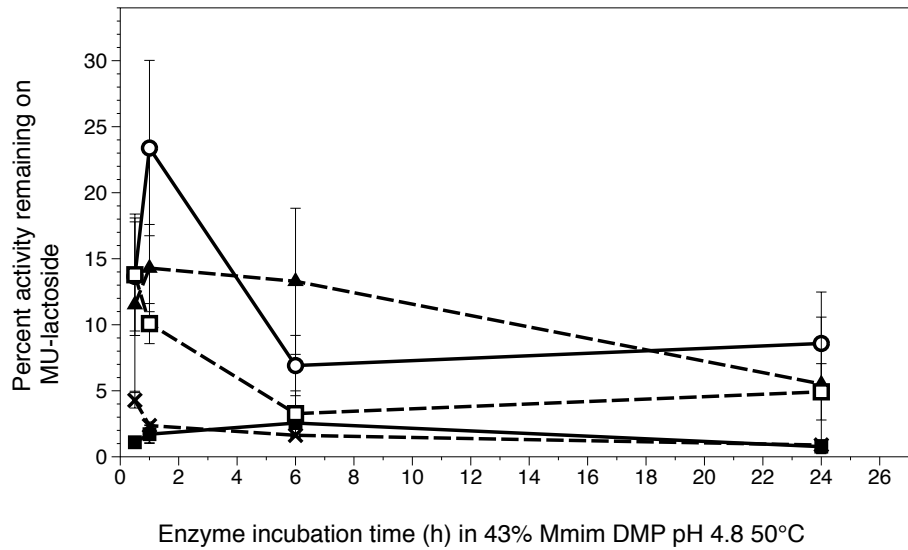
Figure 9. Hydrolysis of 2.5 g/L Emim acetate-pretreated Avicel at 16 h of reaction at 50 °C in 20% Emim acetate pH 4.8 relative to hydrolysis in citrate buffer pH 4.8, with cellulases at 0.2 µM enzyme loading. In this case the enzymes labeled "QC" were treated with glutamine cyclase as described in Methods.

Figure 10. Activity of selected CBHI variants, assayed on MU-lac in 50 mM Sodium acetate at 50 °C after preincubation for varying periods of time in 43% (w/w) Mmim DMP at 50 °C. Closed triangles are 2I13 glutamine cyclase (QC) treated. Open circles are 1M10 QC treated. Closed squares are T.e. Cel7A QC treated. Open squares are T.e. without QC treatment. X's are *T. reesei* Cel7A purified from Celluclast.
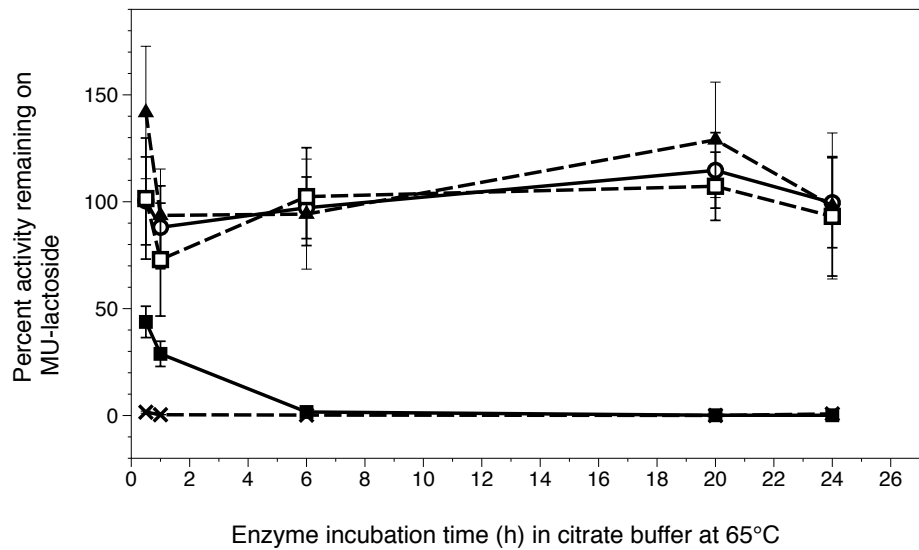
Figure 11. Residual activity of selected CBHI variants at 65 ºC as a function of time. The assay was based on MU-lac at 50 ºC in 50 mM sodium acetate after preincubation for varying periods at 65 ºC. Closed triangles are 2I13 treated with glutamine cyclase. Open circles are 1M10 QC treated.. Open diamonds are 1G21. Open squares are T.e. Cel7A QC treated. Closed squares are T.e. Cel7A without QC treatment. X's are *T. reesei* Cel7A purifed from Celluclast.
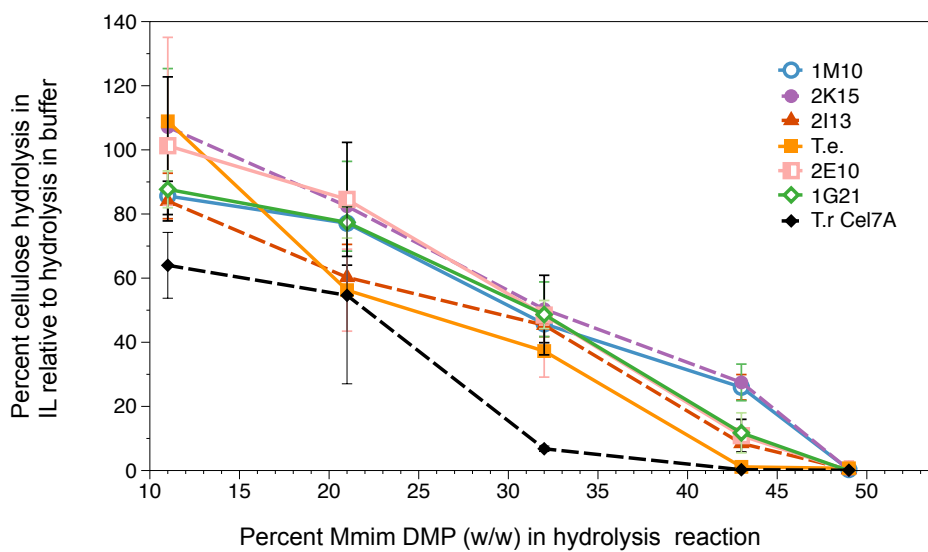
Figure S1a. Avicel hydrolysis of 2.5 g/L IL-Avicel at 16 h of reaction at 50 °C in varying concentrations of Mmim DMP pH 4.8 relative to hydrolysis in citrate buffer pH 4.8, with CBHI variant enzymes at 0.2 μM enzyme loading. The legend on the upper right corresponds to various chimeric mutants.
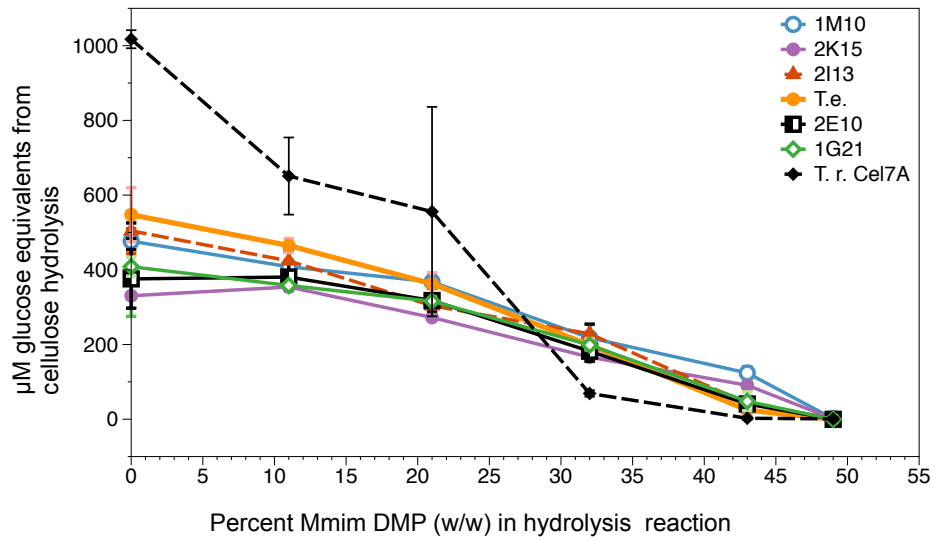
Figure S1B. Avicel hydrolysis of 2.5 g/L IL-Avicel at 16 h of reaction at 50 °C in varying concentrations of Mmim DMP pH 4.8, with CBHI variant enzymes at 0.2 μM enzyme loading. The legend on the upper right corresponds to various chimeric mutants.
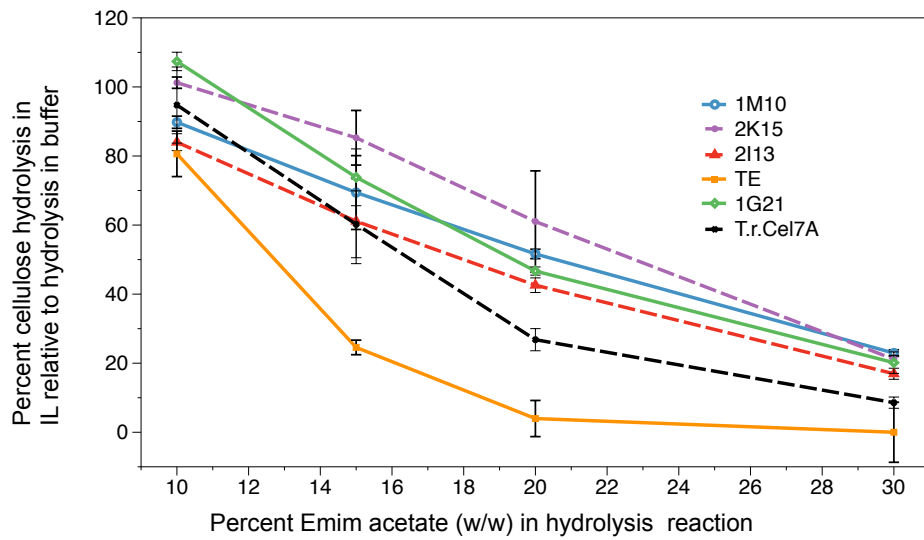
Figure S2a. Avicel hydrolysis of 2.5 g/L IL-Avicel at 16 h of reaction at 50 °C in varying concentrations of Emim acetate pH 4.8 relative to hydrolysis in buferr, with CBHI variant enzymes at 0.2 μM enzyme loading.
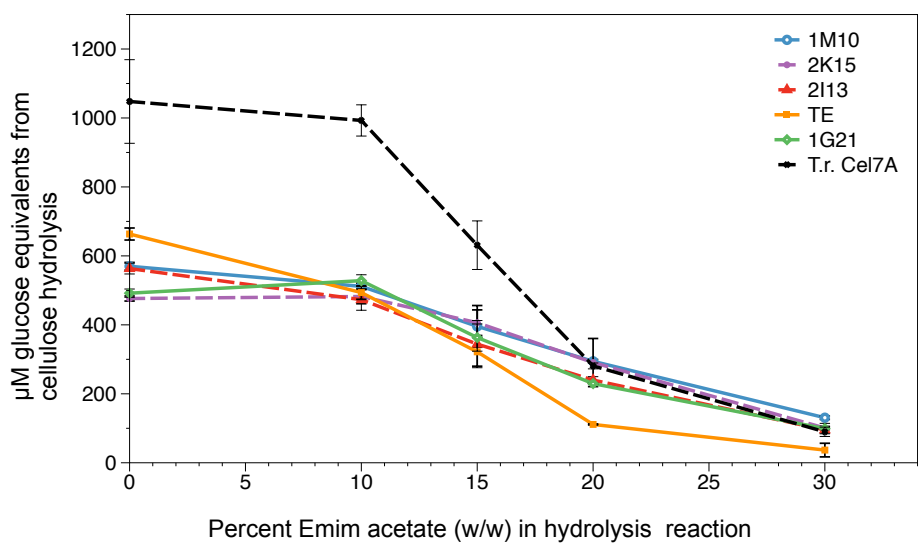
Figure S2b. Avicel hydrolysis of 2.5 g/L IL-Avicel at 16 h of reaction at 50 °C in varying concentrations of Emim acetate pH 4.8, with CBHI variant enzymes at 0.2 μM enzyme loading.
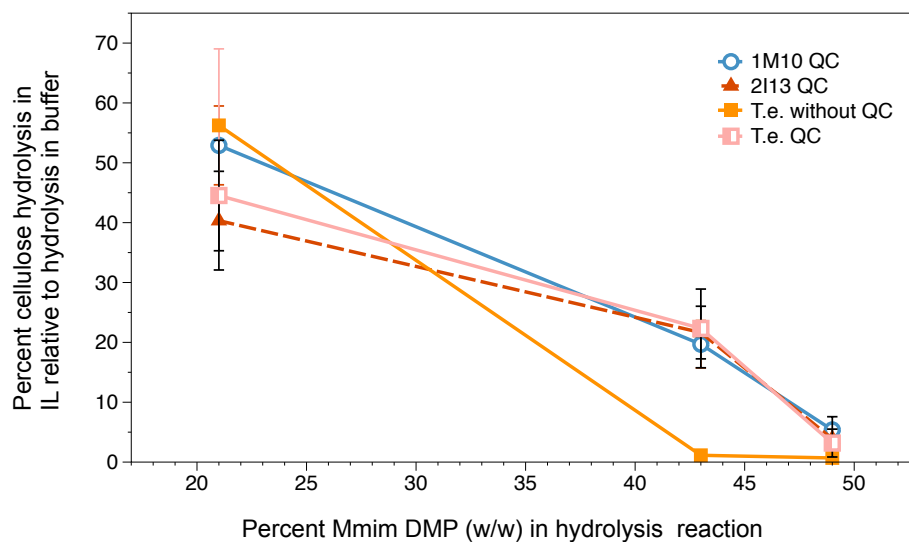
Figure S3a. Avicel hydrolysis of 2.5 g/L IL-Avicel at 16 h of reaction at 50 °C in varying concentrations of Mmim DMP pH 4.8 relative to hydrolysis in citrate buffer pH 4.8, with CBHI variant enzymes at 0.2 µM enzyme loading. The legend on the upper right corresponds to various chimeric mutants. QC refers to glutamine cyclase treatment.3
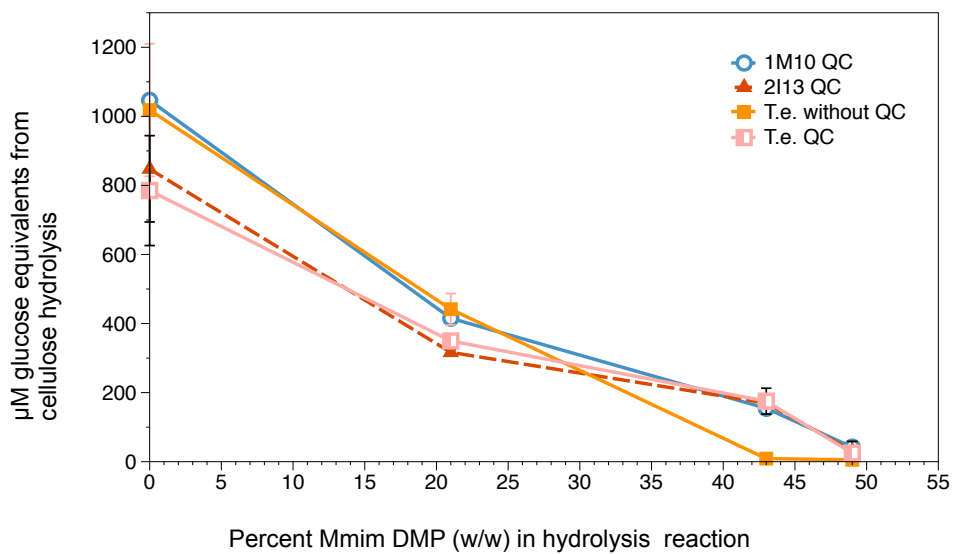
Figure S3b. Avicel hydrolysis of 2.5 g/L IL-Avicel at 16 h of reaction at 50 °C in varying concentrations of Mmim DMP pH 4.8, with CBHI variant enzymes at 0.2 µM enzyme loading. The legend on the upper right corresponds to various chimeric mutants. QC refers to glutamine cyclase treatment.

Figure S4a. Avicel hydrolysis of 2.5 g/L IL-Avicel at 16 h of reaction at 50 °C in varying concentrations of Emim acetate pH 4.8 relative to hydrolysis in buferr, with CBHI variant enzymes at 0.2 μM enzyme loading. QC refers to glutamine cyclase treatment.
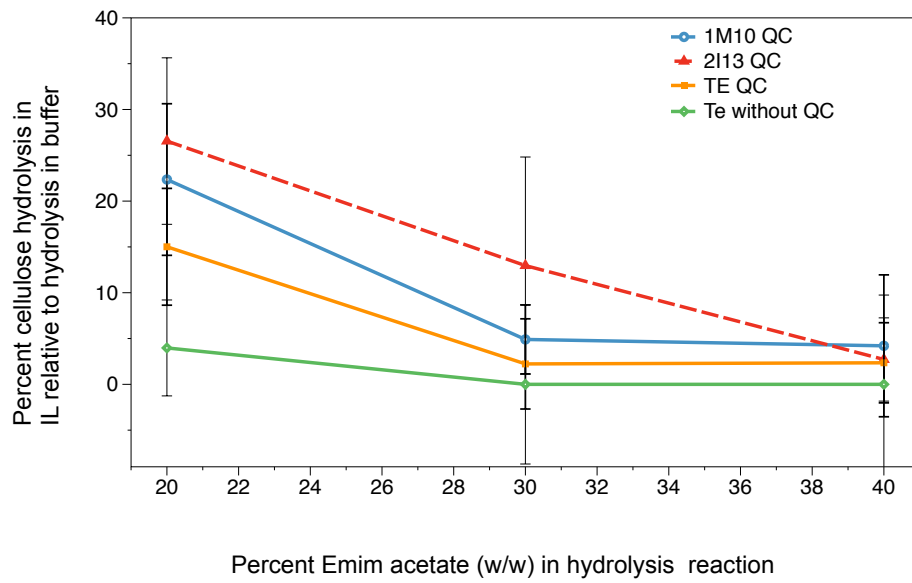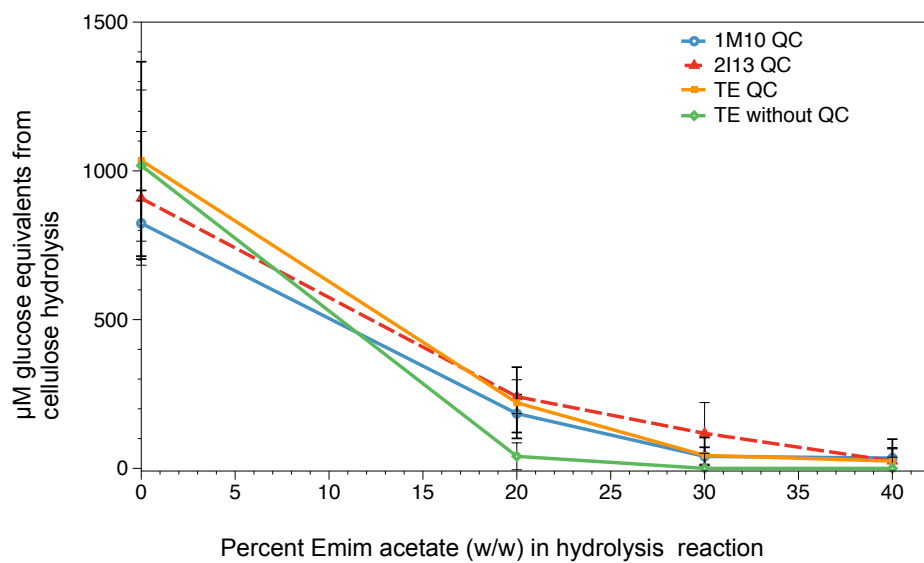
Figure S4b. Avicel hydrolysis of 2.5 g/L IL-Avicel at 16 h of reaction at 50 °C in varying concentrations of Emim acetate pH 4.8, with CBHI variant enzymes at 0.2 μM enzyme loading. QC refers to glutamine cyclase treatment.

Aspergillus terreus
Aspergillus fischerianus
Penicillium chrysogenum
Thermoascus aurantiacus
Aspergillus nidulans
Aspergillus oryzae
Penicillium decumbens
Aspergillus clavatus
Talaromyces emersonii

Table 1: Organisms supplying CBH I catalytic domain genes for shuffling as described by Dana, et. al.[15]

% Mmim DMP (w/w)	Not QC treated Tm (°C) 1M10	Not QC treated Tm (°C) Te	Not QC treated 1st peak Tm (°C) 2I13	Not Qc treated 2nd peak Tm (°C) 2I13	QC treated Tm (°C) 1M10	QC treated Tm (°C) Te	QC treated Tm (°C) 2I13
0	66.3	64.0	65.2	74.6	76.7	74.3	74.6
10	65.1	62.1					
20	62.5	60.8					
30	55.8	52.1					
43	50.1	46.0	45.7	57.6	58.1	56.2	57.7

Table 2: Melting temperature of various CBHI variants in varying concentrations of Mmim DMP measured by differential scanning calorimetry. The two different peaks for the 2I13 refer to whether the sample from had cyclized in storage (2nd peak). QC treated refers to the addition of exogenous glutamine cyclase.

A3V
A6Q
T7K
A8P
N10T
E18K
P22D
P58T
Y60L
N157A
D181N
E183Q

Table 3: Mutations in the variant, "2K15."

A6L	S301K
A8E	I308V
N10V	S311G
P58E	N312K
T59S	Q316N
Y60L	P317S
L73V	N318E
G80A	D320T
V84I	I321W
S87N	T325G
S89D	T438N
K92T	G439P
L105V	T440P
L108M	P441G
Q109E	S442G
N220T	H471M
V222F	

Table 4: Mutations in the variant, "1M10."

```
>1M10_seq
QQAGTLTEEVHPPLTWQECTAPGSCTTQNGAVVLDANWRWVHDVNGYTNCYTGNTWDESLCPDDETCAQNCAVDGADYE
ATYGITSNGDSLTLKFVTGSNVGSRVYLMEDDSTYQIFKLLNREFSFDVDVSNLPCGLNGALYFVAMDADGGVSKYPNN
KAGAKYGTGYCDSQCPRDLKFIDGEANVEGWQPSSNNANTGIGDHGSCCAEMDVWEANSISTAFTPHPCDTPGQTMCSG
DDCGGTYSNDRYAGTCDPDGCDFNPYRMGNTSFYGPGKIIDTTKPFTVVTQFLTDDGTDTGTLKEIKRFYVQNGKVIPN
SESTWSGVGGNSITTEFCTAQKQAFGDTDDFSQHGGLAKMGAAMQQGMVLVMSLWDDYAAQMLWLDSDYPTDADPTTPG
IARGTCPTDSGVPSDVESQSPNSYVTYSNIKFGPINSTFTASNPPGGGTTTTTTTTTSKPSGPTTTTNPSGPQQTMWGQ
CGGQGWTGPTACQSPSTCHVINDFYSQCF
>Te_seq
QQAGTATAENHPPLTWQECTAPGSCTTQNGAVVLDANWRWVHDVNGYTNCYTGNTWDPTYCPDDETCAQNCALDGADYE
GTYGVTSSGSSLKLNFVTGSNVGSRLYLLQDDSTYQIFKLLNREFSFDVDVSNLPCGLNGALYFVAMDADGGVSKYPNN
KAGAKYGTGYCDSQCPRDLKFIDGEANVEGWQPSSNNANTGIGDHGSCCAEMDVWEANSISNAVTPHPCDTPGQTMCSG
DDCGGTYSNDRYAGTCDPDGCDFNPYRMGNTSFYGPGKIIDTTKPFTVVTQFLTDDGTDTGTLSEIKRFYIQNSNVIPQ
PNSDISGVTGNSITTEFCTAQKQAFGDTDDFSQHGGLAKMGAAMQQGMVLVMSLWDDYAAQMLWLDSDYPTDADPTTPG
IARGTCPTDSGVPSDVESQSPNSYVTYSNIKFGPINSTFTASTGTPSGTTTTTTTTTSKPSGPTTTTNPSGPQQTHWGQ
CGGQGWTGPTACQSPSTCHVINDFYSQCF
```

Table S1. Sequences of the T.e. parent gene (including the linker and carbohydrate binding module and the 1M10 variant. Note the N terminal glutamines In both cases.

1. R. P. Swatloski, S. K. Spear, J. D. Holbrey and R. D. Rogers, *J Am Chem Soc*, 2002, 124, 4974-4975.
2. A. P. Dadi, C. A. Schall and S. Varanasi, *Appl Biochem Biotechnol*, 2007, 137-140, 407-421.
3. M. B. Turner, S. K. Spear, J. G. Huddleston, J. D. Holbrey and R. D. Rogers, *Green Chem*, 2003, 5, 443-447.
4. S. H. Lee, T. V. Doherty, R. J. Linhardt and J. S. Dordick, *Biotechnol Bioeng*, 2009, 102, 1368-1376.
5. H. Zhao, G. A. Baker, Z. Song, O. Olubajo, T. Crittle and D. Peters, *Green Chem*, 2008, 10, 696-705.
6. N. Kamiya, Y. Matsushita, M. Hanaki, K. Nakashima, M. Narita, M. Goto and H. Takahashi, *Biotechnol Lett*, 2008, 30, 1037-1040.
7. P. W. Wolski, D. S. Clark and H. W. Blanch, *Green Chem*, 2011, 13, 3107-3110.
8. D. Wilson and D. Irwin, eds. G. Tsao, A. Brainard, H. Bungay, N. Cao, P. Cen, Z. Chen, J. Du, B. Foody, C. Gong, P. Hall, N. Ho, D. Irwin, P. Iyer, T. Jeffries, C. Ladisch, M. Ladisch, Y. Lee, N. Mosier, H. Mühlemann, M. Sedlak, N. Shi, J. Tolan, R. Torget, D. Wilson and L. Xia, Springer Berlin / Heidelberg, 1999, vol. 65, pp. 1-21.
9. T. M. Wood, *Biochemical Society Transactions*, 1992, 20, 46-53.
10. P. N. Brayan, M. L. Rollence, M. W. Pantoliano, J. Wood, B. C. Finzel, G. L. Gilliland, A. J. Howard and T. L. Poulos, *Proteins: Structure, Function, and Bioinformatics*, 1986, 1, 326-334.
11. K. Chen and F. H. Arnold, *Proceedings of the National Academy of Sciences*, 1993, 90, 5618-5622.
12. W. P. C. Stemmer, *Nature*, 1994, 370, 389-391.
13. Y. H. Percival Zhang, M. E. Himmel and J. R. Mielenz, *Biotechnology Advances*, 2006, 24, 452-481.
14. G. L. Moore, C. D. Maranas, K. R. Gutshall and J. E. Brenchley, *Computers & Chemical Engineering*, 2000, 24, 693-699.
15. C. M. Dana, P. Saija, S. M. Kal, M. B. Bryan, H. W. Blanch and D. S. Clark, *Biotechnology and Bioengineering*, 2012, 109, 2710-2719.
16. S. P. Voutilainen, P. G. Murray, M. G. Tuohy and A. Koivula, *Protein Engineering Design and Selection*, 2010, 23, 69-79.
17. P. Heinzelman, R. Komor, A. Kanaan, P. Romero, X. Yu, S. Mohler, C. Snow and F. Arnold, *Protein Engineering Design and Selection*, 2010, 23, 871-880.
18. R. S. Komor, P. A. Romero, C. B. Xie and F. H. Arnold, *Protein Engineering Design and Selection*, 2012, 25, 827-833.
19. J. I. Park, E. J. Steen, H. Burd, S. S. Evans, A. M. Redding-Johnson, T. Batth, P. I. Benke, P. D'Haeseleer, N. Sun, K. L. Sale, J. D. Keasling, T. S. Lee, C. J. Petzold, A. Mukhopadhyay, S. W. Singer, B. A. Simmons and J. M. Gladden, *PLoS ONE*, 2012, 7, e37010.
20. S. Datta, B. Holmes, J. I. Park, Z. Chen, D. C. Dibble, M. Hadi, H. W. Blanch, B. A. Simmons and R. Sapra, *Green Chem*, 2010, 12, 338-345.
21. J. E. Graham, M. E. Clark, D. C. Nadler, S. Huffer, H. A. Chokhawala, S. E. Rowland, H. W. Blanch, D. S. Clark and F. T. Robb, *Nat Commun*, 2011, 2, 375.

22. T. Zhang, S. Datta, J. Eichler, N. Ivanova, S. D. Axen, C. A. Kerfeld, F. Chen, N. Kyrpides, P. Hugenholtz, J.-F. Cheng, K. L. Sale, B. Simmons and E. Rubin, *Green Chemistry*, 2011, 13, 2083-2090.
23. C. M. Dana, personal communication.
24. H. A. Chokhawala, personal communication.
25. W. Humphrey, A. Dalke and K. Schulten, *Journal of Molecular Graphics*, 1996, 14, 33-38.
26. A. Grassick, P. G. Murray, R. Thompson, C. M. Collins, L. Byrnes, G. Birrane, T. M. Higgins and M. G. Tuohy, *European Journal of Biochemistry*, 2004, 271, 4495-4506.
27. M. Kotik and H. Zuber, *European Journal of Biochemistry*, 1993, 211, 267-280.
28. R. J. M. Russell and G. L. Taylor, *Current Opinion in Biotechnology*, 1995, 6, 370-374.
29. C. M. Dana, A. Dotson-Fagerstrom, H. Chokhawala, H. W. Blanch and D. S. Clark, *Biotechnol Bioeng*, 2014, *in press*.
30. A. S. Robinson, V. Hines and K. D. Wittrup, *Nat Biotech*, 1994, 12, 381-384.
31. T.-W. Kim, H. A. Chokhawala, D. Nadler, H. W. Blanch and D. S. Clark, *Biotechnology and Bioengineering*, 2010, 107, 601-611.