

Identification of ionic liquids tolerant cellulases for biomass processing

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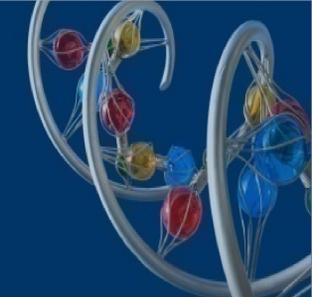
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Biomass pretreatment is the bottle neck in biofuel production

Plant biomass is the most abundant renewable alternative energy resource in nature. The sugars, upon releasing from the biomass, can be converted into biofuels, including ethanol and biodiesel through microbial fermentation. Conventional methods for producing fermentable sugars from biomass require cooking of biomass at high temperature in the presence of acids. These pretreatments increase the cost of biofuel production and cause environmental pollutions. A new next generation pretreatment method, based on ionic liquids (ILs), has been developed to overcome these problems.

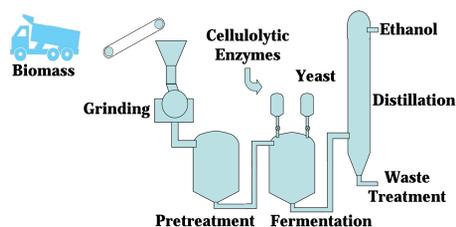


Figure 1. Traditional procedure for making ethanol from cellulosic biomass.

ILs mediated pretreatment requires ILs-tolerant enzymes

ILs are liquid salt at low temperature (<100°C). ILs are strong inhibitors to commercial cellulolytic enzymes. Residual amount of ILs present in pretreated cellulose can cause inhibition to the enzyme activity.

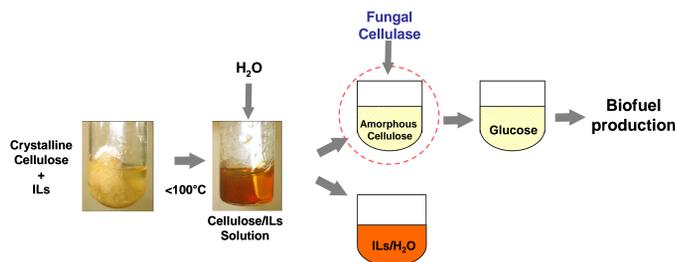


Figure 2. A next generation ILs mediated biomass pretreatment process. ILs are strong solvents that can dissolve cellulose, hemicellulose and lignin. Cellulose can be recovered from ILs solution by adding anti-solvents (such as water). The structure of recovered cellulose is amorphous. Polysaccharides of cellulose pretreated with ILs are exposed and fully accessible to enzymes for digestion.

Where to find ILs tolerant enzymes?

The inhibition of enzyme activity is due to protein denaturation and low water activity in ILs solution. In nature, salt tolerant microorganisms express proteins that are adapted to high salt environment.



Figure 3. *Halorhabdus utahensis* was isolated from Great Salt Lake and sequenced in JGI.

An operon like gene cluster contains large number of putative cellulolytic genes

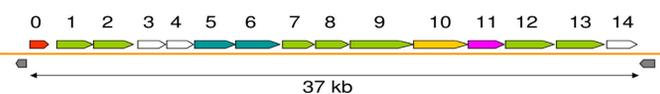


Figure 4. The *Halorhabdus utahensis* genome contains a single gene cluster encoding cellulolytic enzymes with conserved domains. The gene cluster contains 1 sugar specific transcription regulator (in red), 7 cellulase (in green), 2 xylanase (in blue), 1 mannanase (in yellow), 1 pectate lyase (in pink) and 3 proteins with unknown function (in white).

Gene Tag	Interval ID	BLAST hits
Hlu_2386	0	Transcription regulator (sugar-specific)
Hlu_2387	1	Cellulase (GH 5)
Hlu_2388	2	Cellulase (GH 5)
Hlu_2389	3	Hypothetical protein
Hlu_2390	4	Uncharacterized protein
Hlu_2391	5	Beta-1,4-xylanase
Hlu_2392	6	Beta-1,4-xylanase
Hlu_2393	7	Cellulase (GH 5)
Hlu_2394	8	Cellulase
Hlu_2395	9	Cellulase
Hlu_2396	10	Endo-Alpha-mannanase
Hlu_2397	11	Pectate lyase
Hlu_2398	12	Cellulase (GH 5)
Hlu_2399	13	Cellulase (GH 5)
Hlu_2400	14	Hypothetical protein

Table 1. The Gene IDs from 0 to 14 are assigned based on the position of the genes from beginning to the end of the gene cluster. All genes are in same orientation in the cluster. The predicted protein sequences were used to search NCBI NR protein database. The best hit of the BLAST search was assigned to each gene. Sequence similarity between the predicted genes to their best hits is below or close to 50%.

Surface charges of *Hu-CBH1* protein

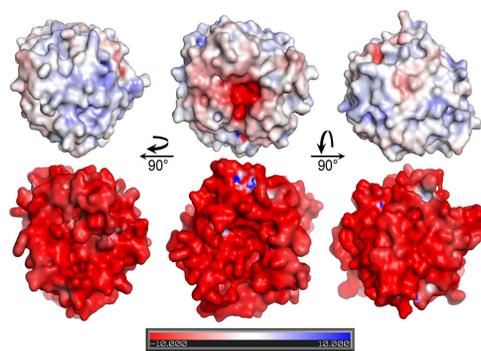


Figure 5. Acidic amino acids are highly enriched in halophilic proteins present in the gene cluster. The *Hu-CBH1* (gene-1) protein surface is extensively covered by negatively charged amino acids. Electrostatics of the cellulase (neutral protein) from *Erwinia chrysanthemi* (PDB:1EGZ; top) and the homology model of the cellulase domain of *Hu-CBH1* (acidic protein) from *Halorhabdus utahensis* (bottom).

The expression of recombinant *Hu-CBH1* proteins in halophilic host

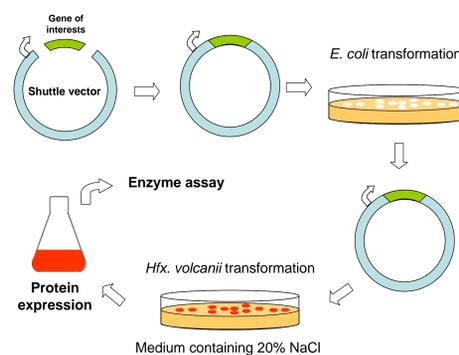


Figure 6. The expression of *Hu-CBH1*. Protein coding region was amplified by PCR and cloned into the *Haloflex volcanii* pJAM202 shuttle vector. The plasmids were first transformed and propagated in *Escherichia coli* cells. Later, *Hu-CBH1*-containing plasmids were transformed into *Hfx. volcanii* cells. Cells were cultured in high salt medium at 40 °C with shaking for 6 to 10 days till the cells were confluent.

Hu-CBH1 enzyme activity in salt

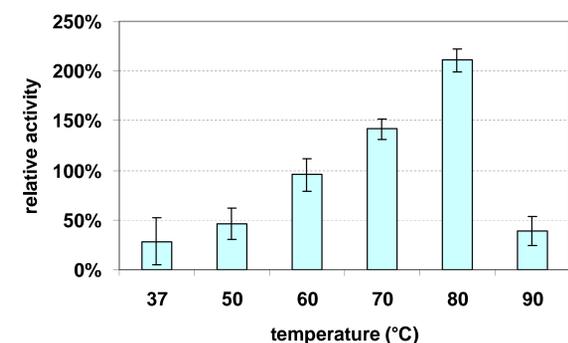


Figure 7. *Hu-CBH1* is a salt tolerant protein and stable at 80 °C in 5M NaCl. A CMC assay was conducted in 5M NaCl buffer and 10 mM Tris-HCl (pH 7.0), at different temperatures for 1 hour. The activity of reaction in 2 M NaCl at 37 °C was set as 100% (data not shown).

Hu-CBH1 activity in the presence of ILs

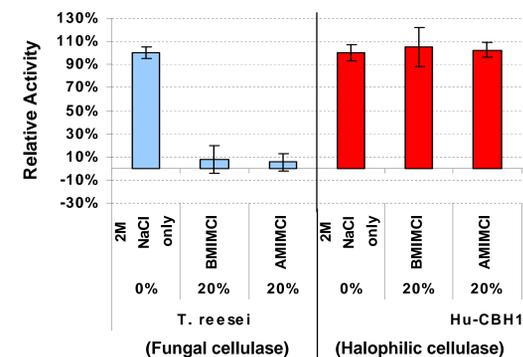


Figure 8. *Hu-CBH1* is resistant to high concentrations of ILs in the presence of salt. *T. reesei* cellulase and the *Hu-CBH1* were incubated with CMC substrate at 37 °C for 1 hour in the presence of 2 M NaCl and 10 mM Tris-HCl (pH 7.0), with or without addition of 20% of [Bmim]Cl and [Amim]Cl. The activity of the reaction performed in 2 M NaCl was set as 100%.

Conclusion

Genome annotation revealed novel halophilic cellulase candidates. Functional analysis demonstrated these are not only salt tolerant, but also salt dependant. Our results suggested that the surface charges of halophilic proteins create a hydration shell that stabilizes these proteins in high salt environment. The same mechanism can be exploited for screening enzymes functioning in high concentration of ionic liquids.

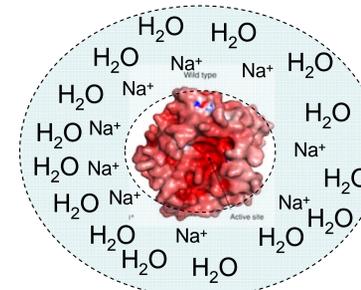


Figure 9. A model of *Hu-CBH1* that explains how surface charges of a halophilic protein may prevent denaturation of protein in extremely high concentration of salt by hydration shell formation.