Identification of ionic liquids tolerant cellulases for biomass processing

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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.

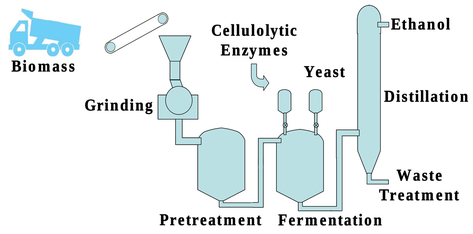

Figure 1. Traditional procedure for making ethanol from cellulosic biomass.

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

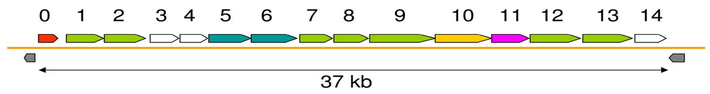

Figure 4. The Halorhabdus utahensis genome contains a single gene cluster encoding cellulolytic enzymes with conserved domains. The gene cluster contains 1 sugar specific transcription regulator (in red), 7 cellulase (in green), 2 xylanase (in blue), 1 mannanase (in yellow), 1 pectate lyase (in pink)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

Locus Tag	Internal ID	BLAST hits
Huta_2386	0	Transcriptional regulator (sugar-specific)
Huta_2387	1	Cellulase (GH 5)
Huta_2388	2	Cellulase (GH 5)
Huta_2389	3	Hypothetical protein.
Huta_2390	4	Uncharacterized protein
Huta_2391	5	Beta-1,4-xylanase
Huta_2392	6	Beta-1,4-xylanase
Huta_2393	7	Cellulase (GH 5)
Huta_2394	8	Cellulase
Huta_2395	9	Cellulase
Huta_2396	10	Endo-beta-mannanase
Huta_2397	11	Pectate lyase
Huta_2398	12	Cellulase (GH 5)
Huta_2399	13	Cellulase (GH 9)
Huta_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%.

Hu-CBH1 enzyme activity in salt

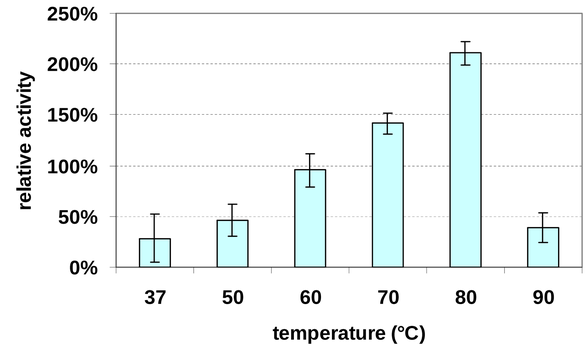

Figure 7. *Hu-CBH1* is a salt tolerant protein and stable at 80 °C in 5M NaCl. A CMC assay was conducted in 5M NaCl buffer and 10 mM Tris-HCl (pH 7.0), at different temperatures for 1 hour. The activity of reaction in 2 M NaCl at 37 C was set as 100% (data not shown).

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 activty.

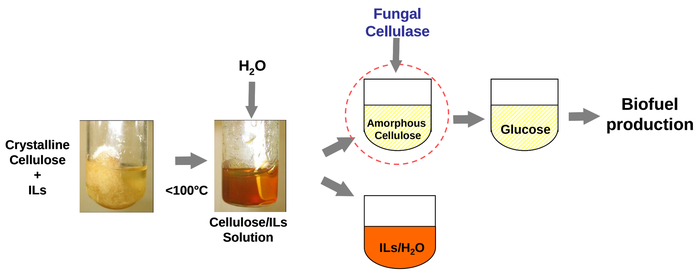

Figure 2. A next generation ILs mediated biomass pretreatment process. ILs are strong solvents that can dissolve cellulose, hemicellulose and lignin. Cellulose can be recovered from ILs solution by adding anti-solvents (such as water). The structure of recovered cellulose is amorphous. Polysaccharides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

Surface charges of *Hu-CBH1* protein

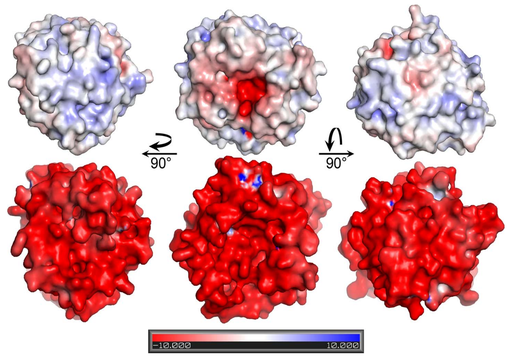

Figure 5. Acidic amino acids are highly enriched in halophilic proteins present in the gene cluster. The *Hu-CBH1* (gene-1) protein surface is extensively covered by negatively charged amino acids. Electrostatics of the cellulase (neutral protein) from Erwinia chrysanthemi (PDB:1EGZ; top) and the homology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

Hu-CBH1 activity in the presence of ILs

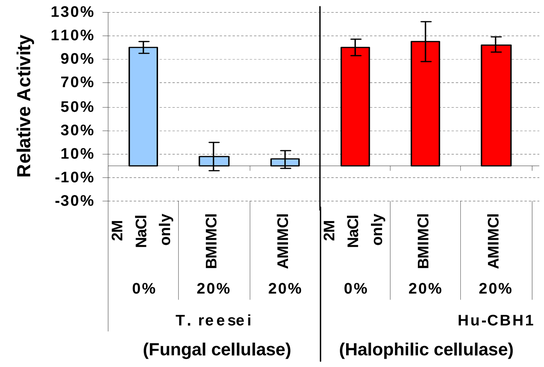

Figure 8. *Hu-CBH1* is resistant to high concentrations of ILs in the presence of salt. *T. reesei* cellulase and the *Hu-CBH1* were are incubated with CMC substrate at 37 °C for 1 hour in the presence of 2 M NaCl and 10 mM Tris-HCl (pH 7.0), with or without addition of 20% of [Bmim]Cl and [Amim]Cl.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

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.

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

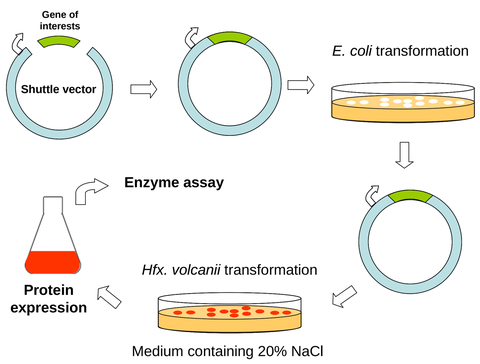

Figure 6. The expression of *Hu-CBH1*. Protein coding region was amplified by PCR and cloned into the *Haloferax volcanii* pJAM202 shuttle vector. The plasmids were first transformed and propagated in *Escherichia coli* cells. Later, *Hu-CBH1*-containing plasmids were transformed into *Hfx. volcanii*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

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.

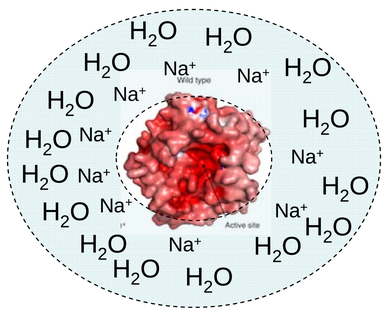

Figure 9. A model of Hu-CBH1 that explains how surface charges of a halophilic protein may prevent denaturation of protein in extremely high concentration of salt by hydration shell formation.

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