2024

Characterization of a Novel Endocellulase Enzyme for Biofuel Optimization

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Characterization of a Novel Endocellulase to Optimize Biofuel Production

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Summary

Currently food crops are used to produce bioethanol production, while plant waste cellulose could be used. However, cellulase enzymes are a limiting factor. We sought to characterize a novel cellulase identified by metagenomic analysis of bovine rumen by the JGI and Hess *et al*. We performed protein expression, genomic analysis, and characterization by SDS-PAGE, CMC and DNS activity assays, and electron microscopy of cellulose degradation. Our data indicates the cellulase is an endoglucanase with an activity of 6.27 cm^2/ug, or 6x higher than commercial cellulase enzymes. These results have implications for creating efficient biofuels from agricultural waste products versus the current methods

Introduction

- Cellulose is the most ample renewable biological resource and has a low-cost energy source based on energy content
- Cellulose is the primary component of the plant cell wall
- The enzyme cellulase breaks down the polysaccharide through hydrolysis at the β-1,4-glycosidic linkages

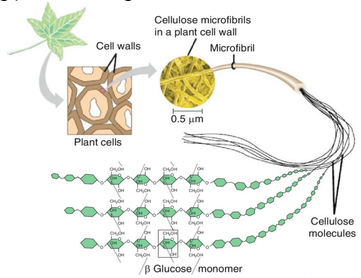


Figure 1. Cellulose the most abundant biomolecule on Earth is composed of glucose monomers. Image https://www.researchgate.net/publication/340136481_Nanocellulose_for_Sustainable_Future_Applications

- Cellulase enzymes break the cellulose polymer chains into glucose monomers which can be fermented to form bioethanol.
- Cellulosic Biofuel allows for renewable energy alternatives to traditional fossil fuel.
- Ruminant cows naturally contain systems to attempt to digest cellulose.
- Metagenomic discoveries attached to plant biomass in cow rumen identified potential biomass-degrading genes from the cow microbiome (Hess *et al*)
- **The objective of this study is to evaluate clones of putative cellulases for their activity compared to commercially available products and identify potential cellulase protein activity found in cow rumen in order to optimize biofuel production.**
- overexpression of cellulase in *E. coli* BL21(*DE3*) with a 6xHis tag
- **Strain [redacted] was evaluated and provided by the Joint genome Institute**

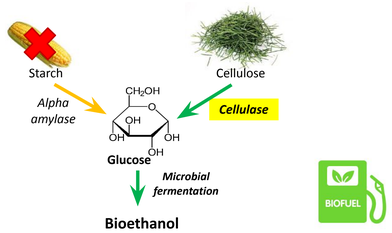
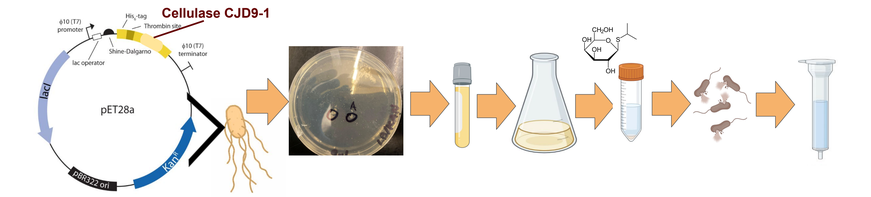


Figure 2. Cellulase enzyme optimization is key for converting bioethanol from feedstock to cellulose. Plant cells wall material is made up of cellulose microfibrils. Cellulose is composed of glucose monomers that with microbial fermentation, produce ethanol which can be used as a biofuel. Currently, food sources such as corn are used primarily for bioethanol. (Hess *et al*)

Methods



$$\text{Cellulase Activity} = \frac{\text{Area Cleared}}{\text{Mass of Protein Added}}$$

Figure 3: Semi-quantitative Carboxymethylcellulose plate assay using congo red detection. Clearing indicates enzyme activity. The control commercial cellulase is shown in the middle. Strains ME9-8 and CJD9-20 are depicted below with enzymatic activity shown in the buffer exchanged and wash samples. 3,5-Dinitrosalicylate Assay measure glucose concentration after digestion of CMC by cellulases..

Results

Cellulase CJD9-1 Bioinformatic Sequence Analysis

Structure of Homologous Cellulase

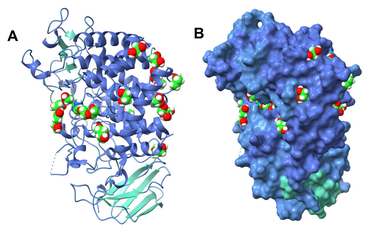


Figure 4. Crystal Structure of PDB Entry 6DHT the highest homology to Cellulase CJD 9-1 putative cellulase. Made in ChimeraX[1,9]. The ligand in 6DHT is di(hydroxyethyl)ether (A) The alpha helices and random coils of the protein are colored in a cornflower blue the beta sheets are colored in cyan. The ligand is colored lime heterochrome. (B) Surface of PDB entry 6DHT. The same color scheme is adopted from image A.

A **Translated Protein Predicted Isoelectric Point and Mass**

Theoretical pI 4.96, Molar Mass 64913 g/mol

B **Blastp Analysis**

Sequence alignment
glycoside hydrolase family 9 protein [Paludibacteraceae bacterium]
Sequence ID: MBO4518690.1Length: **605**Number of Matches: **1**

Score	Expect	Method	Identities	Positives	Gaps
899 bits(2322)	0.0	Compositional matrix	438/591(**74%**)	487/591(82%)	21/591(3%)

C **Protein Data Bank (PDB) Structural Homology Analysis**

Organism	Bacteroides ovatus ATCC 8483
Macromolecule	Xyloglucan-specific endo-beta-1,4-glucanase BoGH9A
Sequence Match	Sequence Identity: **48%**, E-Value: 6.051e-154, Region: 15-567

D

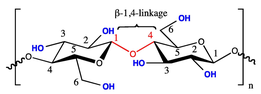


Figure 5. Analysis of genetic sequences for clone CJD9-1. A. Protein mass and isoelectric point prediction. **B.** Translated sequences were compared for alignment in NCBI Blastp and **C.** the PDB. **D.** Cellulose structure with β-1,4 glycosidic linkage.

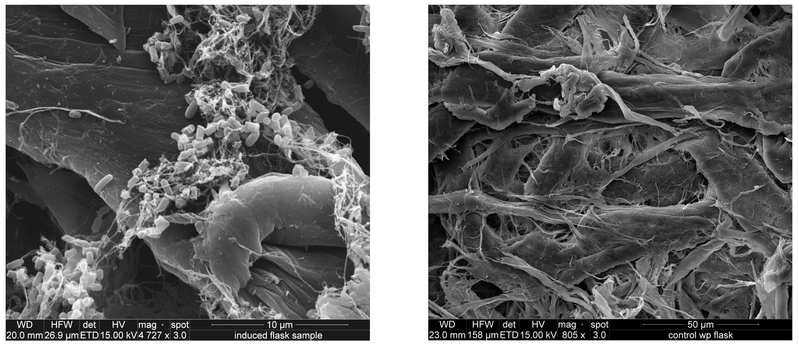


Figure 6. Scanning Electron Microscope (SEM) images of cellulose whatman paper with cellulosic bacteria colonized on surface magnification 4727x (Left) and without bacteria magnification 805x control (Right) on a Quanta 250. Samples were fixed with 3% glutaraldehyde, and successive drying in ethanol 25, 50 , 75, 100%, followed by critical point drying and gold sputter coating.

Results

Molar Mass of CJD-1

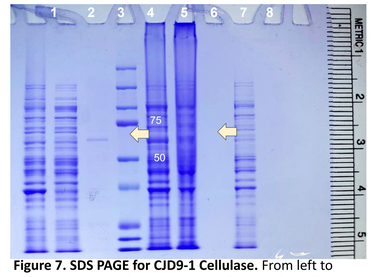


Figure 7. SDS PAGE for CJD9-1 Cellulase. From left to right the lanes are 1. flow through, 2. wash, **3. buffer exchange elution**, 4. kaleidoscope molecular weight ladder, 5 soluble lysate, 6. induced pellet and 8. uninduced pellet.

Table 1. Theoretical and Experimentally Determined Molecular Mass of Cellulase CJD9-1.

Molar Mass Determination	MM (kD)
Theoretical from sequence 1-581	64.9
Theoretical w/out signal sequence 17-581	63.1
Experimental	60.7

Cellulase CJD9-1 Activity is 6x Better

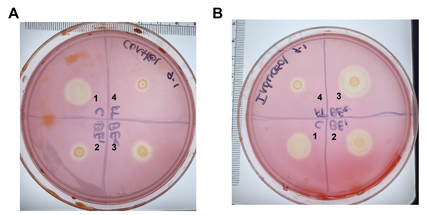


Figure 8. Cellulase Activity Plates. The numbering scheme **1.** Control; **2.** Elution 1; **3.** Elutions 2; **4.** Flow through **Plate A.** Uninduced **Plate B. Induced,** was left to run for 48 hours. Both plates were stained with congo red.

$$\text{Cellulase Activity} = \frac{\text{Area Cleared}}{\text{Mass of Protein Added}}$$

Table 2. Cellulase Activity from Figure 8 B Cellulase Activity Plate Assay

SAMPLE	Activity (cm/ug)
1a. Positive Control (Aspergillus niger Cellulase)	1
2a. Elution (1) from control culture	0.188
3a. Elution (2) from control culture	0.651
4a. Flowthrough from control culture	0
1b. Positive Control (Aspergillus Niger Cellulase)	1
2b. Elution (1) from induced culture	5.63
3b. Elution (2) from induced culture	6.27
4b. Flowthrough	0.291

3,5-Dinitrosalicylate (DNS) Assay

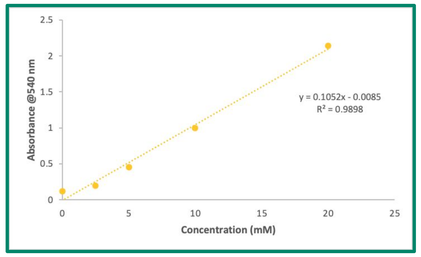


Figure 9. Standard curve from 3,5-dinitrosalicylate assay. Standards were 0, 2.5, 5, 10, and 20 mM glucose solutions.

Table 3. Glucose Concentrations Measured from 3,5-Dinitrosalicylic Assay

	[glucose] (mM)
Buffer Exchanged Elution (I)	1.63
Buffer Exchanged ©	0.95
Media I	0.82
Media C	0.80
Stock Control	2.93

Conclusions

- **Cellulase CJD9-1 a novel cellulases displayed 6x the activity of a commercial cellulase making it a target for enhance biofuel feedstock production**
- The majority of metagenomic cellulases analyzed (10 in this study) did not exceed the activity of the control cellulase (data not shown)
- Sequence analysis indicated homology with the β-1,4 hydrolase family and Enzyme Class EC: 3.2.1.4
- The molar mass of the isolated protein is consistent with the predicted mass with signal sequence, amino acids 1-15, removed.
- Higher activity in CMC assay and lower activity in DNS assay may further substantiates endo-beta-1,4-glucanase or endoglucanase activity of the enzyme as predicted by genetic homology.

Acknowledgments

The research was partially funded by United States Department of Energy Joint Genome Institute Grant . CSP-506518.

This Research was a collaboration through CSUBiotech with Matt Escobar CSUSM.

CIRM also granted use of the bead beater for this research.

References

1. Escobar, Matthew CSU San Marcos. CSUPERB CURES presentation (2019)
2. Hess M, Sczyrba A, Egan R, Kim TW, Chokhawala H, Schroth G, Luo S, Clark DS, Chen F, Zhang T, Mackie RI, Pennacchio LA, Tringe SG, Visel A, Woyke T, Wang Z, Rubin EM. Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. Science. 2011 Jan 28;331(6016):463-7. Boyer, R. F. Modern Experimental Biochemistry 2nd edition (1993) Benjamin Cummings (Redwood City, CA).
3. Al-Jawhari, I.F.H. (2020). Nanocellulose for Sustainable Future Applications. Handbook of Nanomaterials and Nanocomposites for Energy and Environmental Applications. Springer, Cham.