Kinetic Evaluation of Putative Cellulase Enzymes for Cellulosic Biofuel

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Abstract

Cellulose composed of glucose monomers is the most abundant biopolymer on earth, as the primary component of the plant cell wall. It can be broken down with the cellulase class of enzymes, and used to produce cellulosic ethanol as an alternative to fossil fuels. Ruminant derived cellulases can be highly effective and the identities and concentrations of these cellulases produced by ruminant digestive bacteria can vary widely. The enzyme cellulase breaks down the polysaccharide through hydrolysis at the β-1,4-glycosidic linkages. As cellulose is the most ample renewable biological resource and has a low-cost energy source based on energy content, a major obstacle to industrial-scale production of fuel from lignocellulose lies in the inefficient deconstruction of plant material despite a relatively low activity of currently available hydrolytic enzymes. After breaking cellulose polymer chains into glucose monomers using cellulase, microbial fermentation can produce ethanol. Being that cellulase presents an opportunity for green waste production as an alternative to fossil fuels, it is To evaluate potential cellulase protein activity from sequences identified from metagenomic analysis of cow rumen, the sequences were expressed in E. Coli BL21(DE3), induced with Isopropyl-β-D-1-thiogalactopyranoside, harvested with centrifugation and lysed with lysozyme. The protein was then purified with immobilized metal affinity chromatography, buffer exchanged, and analyzed with Carboxymethylcellulose plates and a time-based kinetic assay.

Introduction

- 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
- Cellulose is the most ample renewable biological resource and has a low-cost energy source based on energy content
- After breaking cellulose polymer chains into glucose monomers using cellulase, microbial fermentation can produce ethanol
- Derivation from cellulosic plant material allows the opportunity for renewable energy alternatives to traditional fossil fuel. Plant tissue including grass clippings, crop waste, wood chips, and other organic material can serve as the starting material for cellulosic bioethanol
- Termites and ruminant cows naturally contain systems to attempt to digest cellulose. Metagenomic discoveries attached to plant biomass in cow rumen designate biomass-degrading genes and genomes from microbes
- **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
- Strains ME9-8, CJD8-11, and CJD9-20 were evaluated.

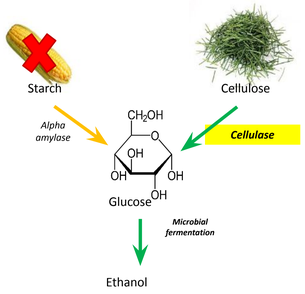

Figure 1: Plant material is made up of cellulose microfibrils which are strands of cellulose molecules. Cellulose is composed of glucose monomers that with microbial fermentation, produce ethanol which can be used as a biofuel.

Methods

A kinetic assay was attempted using 4-Nitrophenyl-β-D-glucopyranoside as a substrate, this is a specialized molecule which contains a sugar moiety attached through a B1,4 glycosidic linkage to Para Nitro Phenol. Upon the cleavage of the Beta 1-4 bond by a cellulase the production of the the yellow colored pNP can be observed using UV-vis spectroscopy. These results were quantified by generating a standard curve of pure pNP solutions. Substrate concentrations were varied so that a representative Lineweaver Burk Plot could be created and a approximate Vmax /Km could be quantified. Methods with a time-based assay were investigated.

4-Nitrophenyl-β-D-glucopyranoside

Figure 2. Kinetic assay reaction

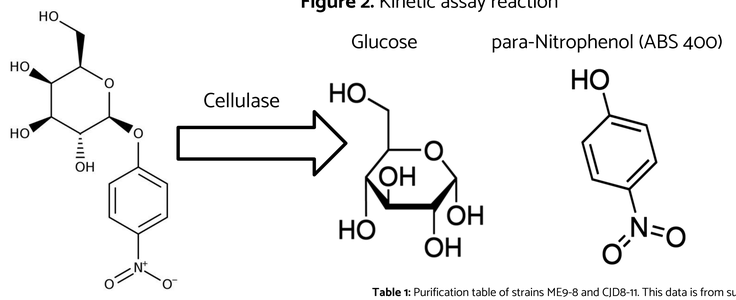

Figure 3: Semi-quantitative Carboxymethylcellulose plate assay using congo red detection. Clearing indicates enzyme activity. The control commercial cellulase is shown in the middle. Strains ME9-8 and CJD9-20 are depicted below with enzymatic activity shown in the buffer exchanged and wash samples.

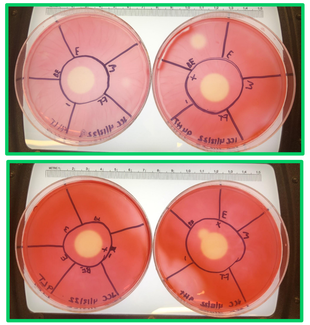

Table 1: Purification table of strains ME9-8 and CJD8-11. This data is from summer of 2021 and represents pHs 7 for the elutions and 8 for after the buffer exchange. This is reflective of successful data that has been collected previously that exceeds the enzymatic activity of this semester.

	Strain/Purification	Diameter (mm)	ug protein	Area/ug protein	*% Efficiency (ABE/+)*
ME9-8	ABE	13.1	2.28	59.1	***103***
	E1	10.9	5.19	18.0	
	(+)	14.8	3.00	57.3	
ME9-8	ABE	16.0	8.70	23.1	***45.0***
	E1	12.2	3.81	30.7	
	(+)	14.0	3.00	51.3	
CJD8-11	ABE	8.00	0.84	59.8	***100.0***
	E1	0	3.36	0	
	(+)	15.1	3.00	59.7	
CJD8-11	ABE	10.2	3.42	23.9	***46.6***
	E1	0	2.46	0	
	(+)	14.0	3.00	51.3	

Results

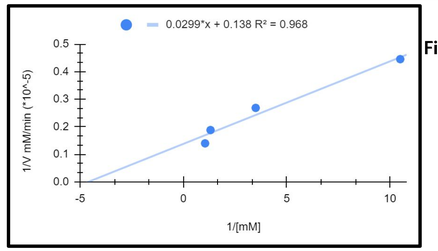

Figure 4. Line Weaver Burke plot

A Lineweaver-Burke plot was generated using commercial Aspergillus Niger cellulase. Novel strains isolated from the cow's rumen showed too little activity to properly quantify.

Results

- Previous semesters' work have demonstrated activity for strains ME9-8 and CJD9-8 at various pHs. Recent lack of expression likely stems from systemic errors possibly with IPTG which is used for induction.
- The vast majority of cellulases did not exceed the activity of the control, let alone display activity with the semi-quantitative CMC plates.
- With the exception of CJD9-20, these novel cellulases have not displayed consistent or considerable activity.
- ME9-8 demonstrated more activity than the other strains at pHs 6-8.
- Kinetic experiments for commercial Cellulase showed an approximate Vmax of 7.25*10^-5mM/min maximal reaction rate or velocity of an enzymatically catalyzed reaction when the enzyme is saturated with its substrate. The experimental Km was determined to be approximately 0.216mM as the inverse measure of affinity.

Conclusions

- Errors with induction demonstrated little enzymatic activity and protein expression
- The activity demonstrated in strain CJD9-20 demonstrated basal expression
- This is the first semester that kinetic assays have been conducted on these novel cellulases so the procedure was greatly modified in various stages
- The kinetic activity demonstrated by enzyme indicates that reactions likely need to be carried out over a longer time frame with more cellulase and substrate to produce reputable data.

Future Work

- Larger cultures must be prepared in order to evaluate the kinetic activity with enough protein
- Experiments with fresh IPTG should be done in order to be certain of expression.
- Focus more efforts on specific strains that have previously shown activity such as ME9-8 and CJD9-8
- An end point based assay instead of a time based assay may deliver better results but requires more protein.

Acknowledgments

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