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Expression, Purification, and Crystallization of an Endoxylanase from *Bacteroides Vulgatus*

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ABSTRACT

Sustainable sources of energy are growing in demand as fossil fuels are rapidly expended. One such energy source is fuel ethanol generated from the fermentation of plant biomass by engineered bacterial biocatalysts. The creation of a biocatalyst capable of converting nearly any plant matter to fuel ethanol requires the identification of novel enzymes capable of degrading specific carbohydrate polymers and cloning these enzymes into a bacterial host. This study seeks to structurally characterize a novel xylanase of glycosyl hydrolase family 30 (GH30) from *Bacteroides vulgatus*, a bacterium found in the human gut microbiome, via x-ray crystallography. The gene for *B. vulgatus* GH30 endoxylanase (BvGH30) was cloned into a pET 28b prokaryotic expression vector which was used to transform a culture of *Escherichia coli*, and the resulting bacterial strain was used to express the cloned BvGH30 gene. The recombinant protein produced was then purified to homogeneity via Ni²⁺-Metal Chelating Affinity Chromatography (MCAC) as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The purified protein was concentrated to 10 mg/mL and used to screen for solution conditions that promoted crystal growth by sparse matrix screening in hanging drop, vapor-diffusion plates. Single rectangular crystals of defined morphology (less than 0.1mm in length) were obtained in 0.2M (NH₄)₂HPO₄, 20% (w/v) PEG 3350, pH 8.0 and large numbers of smaller rectangular crystals were obtained in 0.1M Citric acid, 0.8M (NH₄)₂SO₄, pH 4.0. Grid screening around these two conditions will be employed to increase crystal size and all crystals larger than 0.1mm in length will be subjected to x-ray diffraction analysis.

INTRODUCTION

As non-renewable energy resources are depleted at an alarming rate, and some researchers predicting fossil fuel reserves to be consumed within the next hundred years (Shafiee & Topal, 2009), alternate, sustainable sources of energy are being sought with increasing urgency. Plant biomass has been used for over a decade as one such renewable energy source, however its use is not without problems. Specifically, current implementation of plant biomass for energy production has revolved around the utilization of “low hanging fruit” or the easiest to use components of plant tissue, such as corn kernels or sugar cane juice. The easily fermentable glucose found in these materials is converted to fuel ethanol by microbial biocatalysts. Unfortunately, the diversion of these materials from animal and human food processing streams ultimately

results in decreased availability of food for lesser-developed countries (Runge & Senauer, 2007). In order to minimize the impact of using possible foodstuffs as substrate for fuel ethanol production, researchers have begun turning their attention to seldom-used polysaccharide materials currently discarded as waste. Materials like sugar cane bagasse and corn stalks contain up to 30% hemicellulose. Hemicellulosic biomass contains polymers of xylose (xylans) and galacturonic acid (pectins). The development of biocatalysts that can ferment these materials relies upon the discovery and characterization of enzymes capable of degrading the polymers to their component sugars, which can then be fermented to ethanol by the microbes.

Glycosyl hydrolases are enzymes that degrade polysaccharide polymers via the addition of a water molecule to the glycosidic

bond between monomeric units. Xylanases are enzymes that hydrolyze xylans, hemicellulosic polymers found in plant cell walls that are composed of β -xylose monomers linked via 1,4 glycosidic bonds. At present, endoxylanases are found in 3 distinct families of the glycosyl hydrolases (GH): GH family 10, GH Family 11 and GH Family 30. Each family of xylanase generates unique products during the hydrolysis of xylan substrates. Members of GH30 hydrolyze the glycosidic bond between glucuronic acid (GA) residues one position from the non-reducing terminus at the β -1,4 bond and requires the presence of α -1,2-linked 4-O-methyl-D-glucuronic acid substituents for substrate recognition and anchoring by binding pocket residues of the xylanases (Hurlbert & Preston 2001; St John *et al.* 2011, Urbániková *et al.*, 2011).

Previous research suggests that binding pocket residues most significant to the catalytic function in glycosyl hydrolases are aspartate and/or glutamate (Davies & Henrissat, 1995). More recent studies have identified other critical catalytic residues, such as arginine, to be involved in optimal substrate positioning for hydrolysis via interaction with negatively charged hydroxyl groups of GA appendages, ultimately increasing catalysis efficiency (St John *et al.*, 2014; Urbániková *et al.*, 2011). However, a study of *Clostridium papyrosolves* of GH30 (CpXynA) by St John *et al.* (2014) found that while typically conserved, arginine is not critical to enzyme function. Although the lack of arginine, specifically in the β 8- α 8 loop, alters the shape of the binding pocket of the enzyme and lessens catalysis efficiency, the adjusted structure allows active site recognition of other hemicellulosic substrates such as xylooligosaccharides and arabinoxylans.

As part of our efforts to characterize novel GH30 xylanases, we are attempting to determine the x-ray crystallographic structure of 3 enzymes identified by bioinformatic searches of genomic databases. We have identified an endoxylanase of GH30 from *Bacteroides vulgatus* (BvGH30), an organism found in the human gastrointestinal tract. The amino acid sequence of the GH30 xylanase from *B. vulgatus* reveals the absence of the essential arginine residue in

the β 8- α 8 loop region. Thus, in accordance with prior research, *B. vulgatus* is most likely able to hydrolyze GA, and possibly arabinoxylan and neutral xylooligosaccharides, though inefficiently (St John *et al.*, 2014). In this study, we seek to determine the 3-dimensional structure of the endoxylanase in order to understand the relationship between its structure and function of interest to biocatalysts development.

MATERIALS AND METHODS

Bacterial Transformation of E. coli Rosetta 2

To grow transformed bacterial colonies, 2 mL of *Bacteroides vulgatus* GH30 pET 28b plasmids were inserted into a micro-centrifuge tube containing 100 mL of *Escherichia coli* Rosetta 2. The centrifuge tube was then placed on ice for 10 minutes, into a water bath at 42° C for 2 minutes, and finally back on ice for 10 min. 500 mL of Luria-Bertani broth media was then added to the centrifuge tube, which was then incubated with shaking for 45 min at 37° C. After incubation, 100 mL of the aliquot was spread onto a plate containing agar that had been infused with antibiotics kanamycin and chloramphenicol. The plate was finally incubated at 37° C for 24 hours. Single colonies were cultured into flasks that each contained 100 mL of Luria-Bertani broth media and 100 mL of kanamycin and chloramphenicol. The flasks were incubated with shaking for 24 hours at 37° C. 1L flasks containing 1L of fresh media and 100mL of each antibiotic were then inoculated with 10mL of culture and incubated at 37° C for 3 hours.

Expression of BvGH30

Once cultures reached optimal densities of 0.6 absorbance at 600nm, 0.1mM of Isopropyl β -D-1-thiogalactopyranoside was added to induce protein production. The cultures were incubated at 37° C with shaking for 24 hours. Cultures were transferred into 1L centrifuge tubes and centrifuged at 4,500 rpm for 10 minutes.

Cell Lysis of E. coli Rosetta 2

Following the initial centrifugation, the supernatant was drained and the cell pellet was mixed with 5mL/g of lysis buffer. Sonification was conducted for 30 minutes at pulses of 15sec

per 45 seconds at 70 amperes. The mixture was then centrifuged at 15,000 rpm for 45 minutes, and the supernatant was collected.

Purification of BvGH30

Supernatant of cell lysis centrifugation was collected for chromatogram analysis with Ni²⁺-Metal Chelating Affinity Chromatography by injections of 50 mL. The UV detector read 280 nm to identify protein presence, and purification was facilitated by a 500mM imidazole buffer wash. The pure BvGH30 was then transferred into 22mm dialysis tubing with a MWCO of 10 kDa and dialyzed with 25mM HEPES and 150mM NaCl buffer over 48 hours. Protein was finally spin-concentrated to 10 mg/mL.

SDS-PAGE of Samples from Each Step of Purification

Of each product of the purification process, 20 mL of samples were mixed with 5 mL of 5X SDS-PAGE loading dye. The samples were heated on a heating block for 30 seconds. Run at 70 V, Precision Plus Protein™ Dual Color Standard by Bio-Rad was loaded onto the gel at volume 5 mL along with samples

loaded at 20 mL. Electrophoresis was ceased when the leading band of the standard reached the front edge of the gel. The gel was then stained for 24 hours and de-stained.

Crystallization of BvGH30

Concentrated pure protein was subjected sparse matrix screening of 280 conditions within Hampton Research Crystallization Screens as well as 50 by a Sigma Aldrich Biochemika Crystallization kit were employed utilizing hanging drop, methods. 750 mL of solution of each condition was injected into 24 well plates were used, then 2 mL of well solution and 2 mL of protein were micro-pipetted onto a coverslip that was finally inverted and sealed onto the well. Crystals were found to grow in 0.2M Ammonium phosphate dibasic pH 8.0 with 20% w/v Polyethylene glycol 3,350 as well as 0.1M Citric acid pH 4.0 and 0.8M Ammonium sulfate. In order to optimize the crystal size and regularity, a grid screen made of Ammonium Phosphate at basic pH's and Polyethylene glycol 3,350, successfully yielding crystals.

RESULTS

BvGH30 Purification

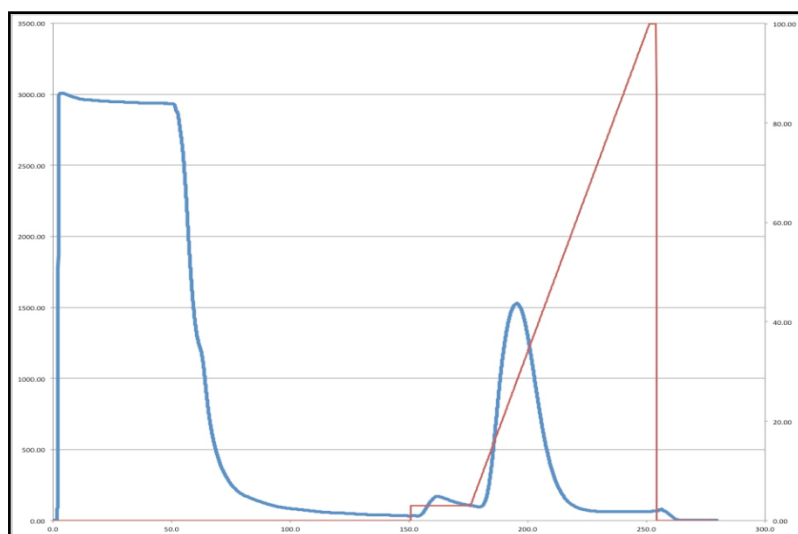
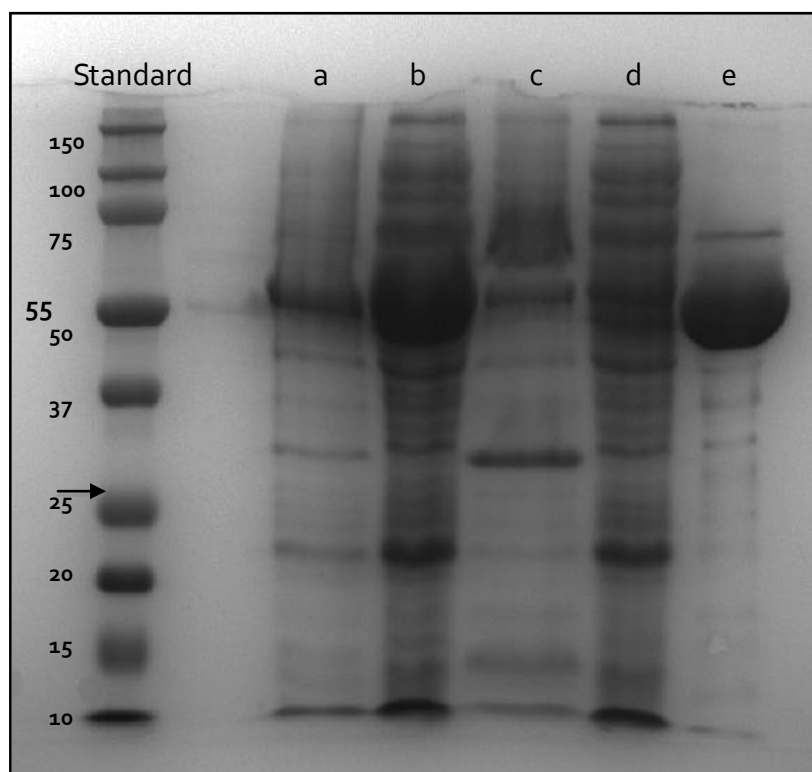


Figure 1: Chromatogram from Purification of BvGH30.

The blue peak of the chromatogram indicates where protein was detected upon elution from the column with 500 mM imidazole buffer. The eluted protein peak was then analyzed via SDS-PAGE gel to confirm identity and successful purification.

SDS PAGE of Each Step of Purification



Lane *a* contains a sample of the cell pellet from the initial centrifugation. Lane *b* contains a sample of the lysate supernatant of the pellet from the second centrifugation. Lane *c* contains a sample from cell pellet of the second centrifugation. Lane *d* contains a sample from the flow through from chromatography. Lane *e* contains a sample from the peak fraction from chromatography.

Figure 2: SDS-PAGE gel of Each Step of Purification of BvGH30.

Crystallization: Sparse-Matrix Screening

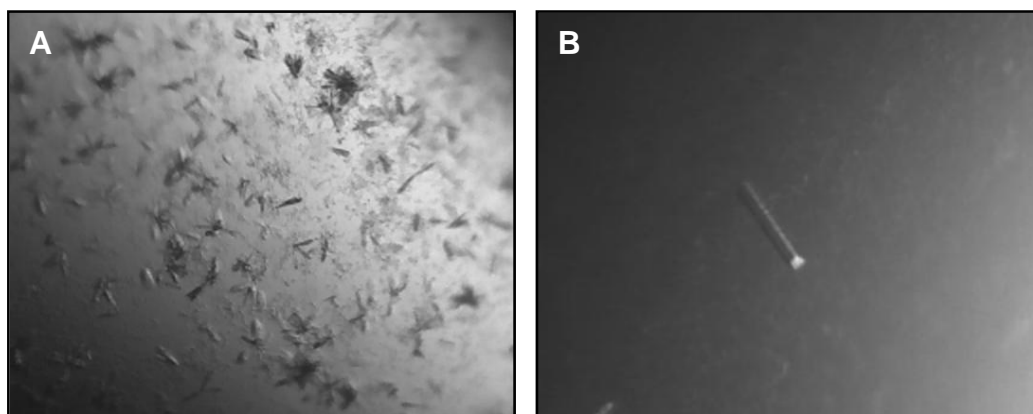


Figure 3: Initial Crystallization Trials of BvGH30

Figure 3A depicts crystals formed with 0.1M Citric acid pH 4.0 and 0.8M Ammonium sulfate. Figure 3B depicts crystals formed with 0.2M Ammonium phosphate dibasic pH 8.0 with 20% w/v Polyethylene glycol 3,350.

Crystallization: Grid-screening

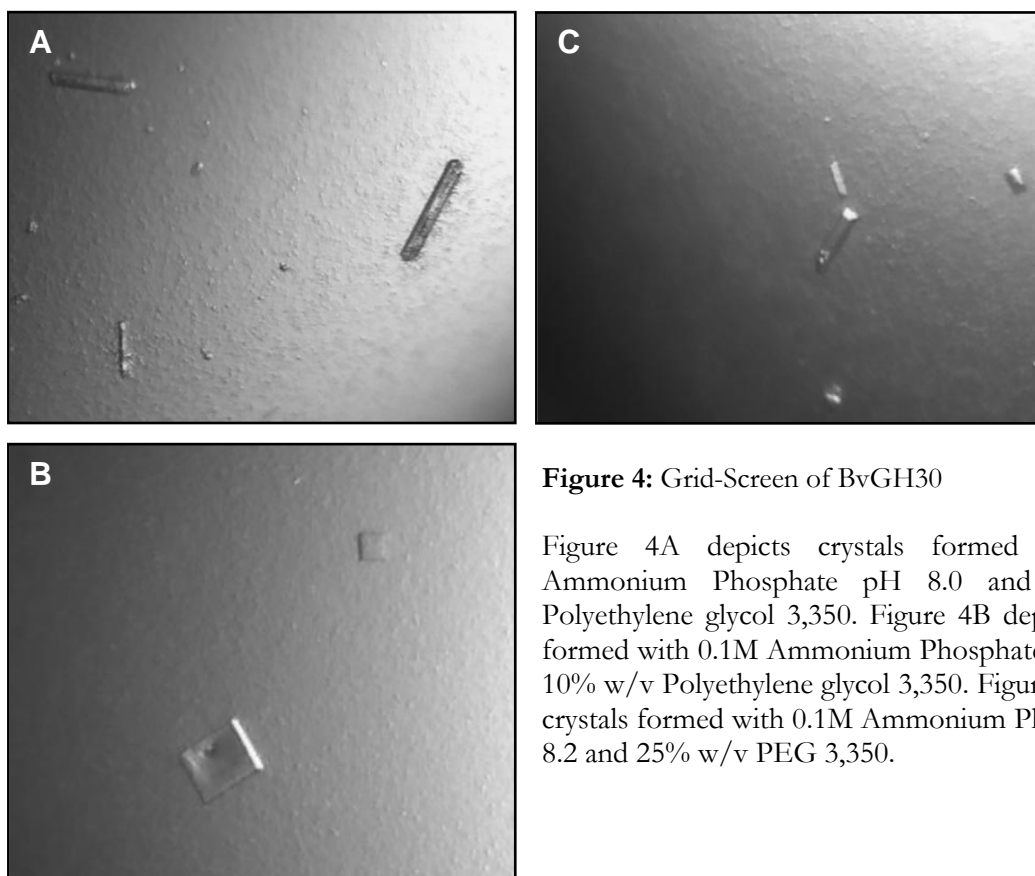


Figure 4: Grid-Screen of BvGH30

Figure 4A depicts crystals formed with 0.1M Ammonium Phosphate pH 8.0 and 20% w/v Polyethylene glycol 3,350. Figure 4B depicts crystals formed with 0.1M Ammonium Phosphate pH 8.4 and 10% w/v Polyethylene glycol 3,350. Figure 4C depicts crystals formed with 0.1M Ammonium Phosphate pH 8.2 and 25% w/v PEG 3,350.

DISCUSSION

Pure protein was successfully eluted at 200 mL with a wash of 500mM imidazole buffer via its interception of hexa-histidine tags on BvGH30 (Figure 1). Purity was confirmed by SDS-PAGE where only protein of molecular weight ≈ 55 kDa, the weight of BvGH30, was revealed (Figure 2). Sparse matrix crystallization screens ensued and produced crystals under two conditions: in 0.2M $(\text{NH}_4)_2\text{HPO}_4$, 20% (w/v) PEG 3,350, pH 8.0 (Figure 3A) as well as 0.1M Citric acid, 0.8M $(\text{NH}_4)_2\text{SO}_4$, pH 4.0 (Figure 3B). The major trend identified between the two conditions is the presence of polyatomic anions: phosphate and sulfate. Thus, 0.1M $(\text{NH}_4)_2\text{HPO}_4$ was used in grid screening, along with varied basic pH's as well as concentrations of PEG 3,350. Crystals were obtained from 0.1M $(\text{NH}_4)_2\text{HPO}_4$ pH 8.0, 20% w/v PEG 3,350 (Figure 4A), 0.1M $(\text{NH}_4)_2\text{HPO}_4$ pH 8.2, 25% w/v PEG 3,350 (Figure 4B), and 0.1M

$(\text{NH}_4)_2\text{HPO}_4$ pH 8.4, 10% w/v PEG 3,350 (Figure 4C). Although this grid screen did not yield crystals of adequate size ($>0.1\text{mm}$), significance of polyatomic anions in crystallization of BvGH30 was confirmed, upon which future work can be conducted.

FUTURE WORK

As the first round of grid screening produced crystals less than 0.1 mm in length, further crystal scoring as well as grid screening expanding upon conditions that employ polyatomic anions must be conducted. Once protein crystals of adequate size and regularity are acquired, crystals will be screened for x-ray diffraction data collection and the three-dimensional structure of BvGH30 will be determined.

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