Characterization of a xylan-degrading enzyme from hyperthermophile *Fervidibacter sacchari*

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Overview

Fsa15405Xyn was predicted to be a cytoplasmic glycoside hydrolase family 3 (GH3) enzyme from the hyperthermophilic bacterium Fervidibacter sacchari. We show that Fsa15405Xyn is an exo-β-1,4-xylanase. Computed structure models and native polyacrylamide gel electrophoresis show that Fsa15405Xyn is a dimer. Its temperature and pH optima are 90 °C and pH 4.5-7.5; however it degrades within an hour at 100 °C. The K_M is 85.4 μM; the V_{max} is 295.7 μM/min; and the K_{cat}/K_M is 0.399 $\mu M^{-1}s^{-1}$. Exo-β-1,4-xylanase activity was confirmed by strong activity on 4-nitrophenyl-β-xylobioside and the production of xylose monomers from xylan. This suggests Fsa15405Xyn is an important cytoplasmic component of an endo-xylanase/exo-xylanase system that degrades xylan and other xylose-containing polymers completely to xylose monomers.

Introduction

The hyperthermophile *Fervidibacter sacchari* was first predicted based on single-celled genomes (Rinke et al., 2013) from Great Boiling Spring (GBS) in Gerlach, Nevada (**Figure 1a**) and has since been isolated (**Figure 1b**) (Nou et al., 2024). F. sacchari is the first isolated bacterium from the class, *Fervidibacteria*, and the sixth from the phylum, *Armatimonadota*. The phylum specializes in degrading polysaccharides, with *F. sacchari* itself encoding 117 predicted glycoside hydrolases (GHs) (Nou et al., 2024). After synthesizing, expressing, and screening these predicted GHs, one of the enzymes, designated Fsa15405Xyn, was shown to degrade xylan (**Figure 1c**). Proteomics from *F. sacchari* in the presence of eight polysaccharides show that Fsa15405Xyn has low overall expression. In mixed culture of *F. sacchari* with other microbes from Great Boiling Spring, Fsa15405Xyn is one of only 34 proteins expressed. Hidden Markov models predicted Fsa15405Xyn to belong to glycoside hydrolase family 3 (GH3), which includes xylanases.

Here, we characterize Fsa15405Xyn in order to better understand the ecophysiology of *F. sacchari* and its enzyme systems to degrade xylose-containing polysaccharides. Since *F. sacchari* occupies a unique niche as the only known aerobic hyperthermophile within geothermal springs (an early Earth-like environment), studying it and enzyme systems that are critical for its lifestyle is relevant to the NASA Science Mission Directorate's goals to better understand the origin and early evolution of life and life in extreme environments.

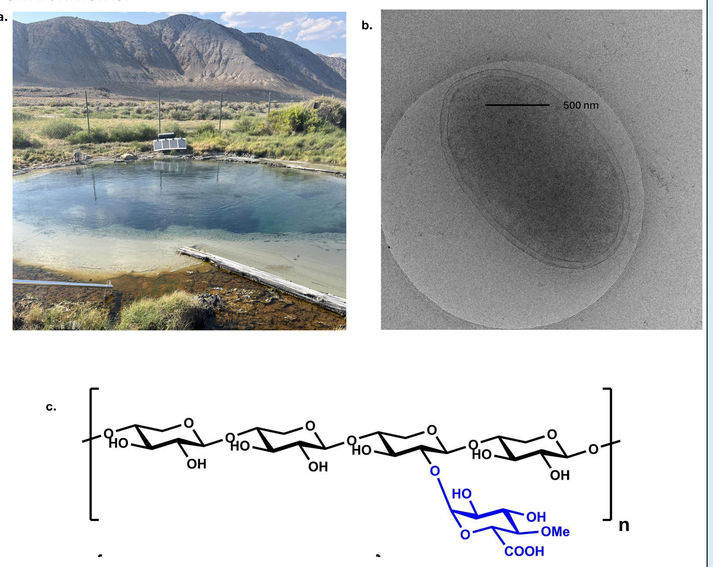

Figure 1: a. Great Boiling Spring, Gerlach, NV, USA; **b.** Cryogenic Electron Microscopy (Cryo-EM) image of *Fervidibacter sacchari;* **c.** Structure of Beechwood Xylan (Megazyme)

Methods

1. Fsa15405Xyn was synthesized, ligated into the pET21b plasmid, and cloned in T7 Express *Escherichia coli* cells. *E. coli* cells were grown on Luria-Bertani (LB) agar plates with 100 μg/mL ampicillin overnight. Colonies were transferred to liquid LB broth with ampicillin and grown to an optical density of 0.6 before the expression of Fsa15405Xyn was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated overnight at 37 °C.
2. The cells were centrifuged to form a pellet and the broth discarded. The pellet was resuspended in lysis buffer. Lysozyme (5 μL/mL) was added to the buffer and cells were lysed by sonication. The lysed cells were once again centrifuged down into a pellet. The supernatant was collected. To purify the enzyme of interest, the supernatant was heated to 80 °C for 30 minutes. The solution was centrifuged again. The supernatant was collected and used in subsequent experiments.
3. The enzyme was visualized using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to determine purity of the enzyme. The enzyme was also visualized by native-PAGE to determine whether it existed as a multimer in *E. coli*.
4. Computed Structural Models (CSMs) were generated using the AlphaFold 2 plug-in on Chimera X.
5. Temperature range and optimum were determined by mixing Fsa15405Xyn and its substrate, beechwood xylan (in a 1% solution), for 1 hour at various temperatures (4 °C to 110 °C). A 3,5-dinitrosalicylic (DNS) acid assay was used to quantify reducing sugars.
6. pH optimum and range were determined by mixing Fsa15405Xyn with its substrate in buffers at various pH values (3.0 to 11.0) for 1 hour at 90 °C and quantifying activity using the DNS assay.
7. Thermostability was determined by incubating Fsa15405Xyn at various temperatures (70 °C to 100 °C) for 1 hour before adding the substrate. Then, the reaction was allowed to proceed for 1 hour at 90 °C and pH 7.0 and quantifying enzyme activity using the DNS assay.
8. Enzyme kinetics were determined by mixing Fsa15405Xyn with 4-nitrophenyl-β-xylobioside at various concentrations (1.25 mM to 20 mM) for 30 minutes at 90 °C and pH 7.0. The reaction was quenched using 20% disodium phosphate. Activity was quantified by measuring absorbance at 400 nm.
9. Ultra-performance liquid chromatography followed by tandem mass spectrometry (UPLC-MRM-MS) was used to quantify sugars released from beechwood xylan by Fsa15405Xyn under optimal conditions for 1 hour.

Results

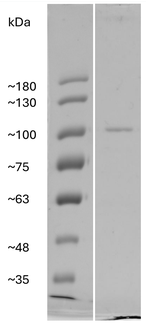

Figure 2: SDS-PAGE. The recombinant enzyme is estimated at ~82 kDa. The enzyme was estimated to be ~95% pure.

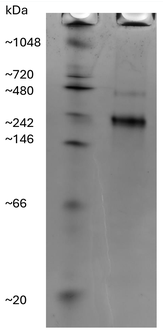

Figure 3: Native-PAGE. The recombinant enzyme forms a band at ~200 kDa. Given that Fsa15405Xyn is ~82 kDa, this suggests a dimer.

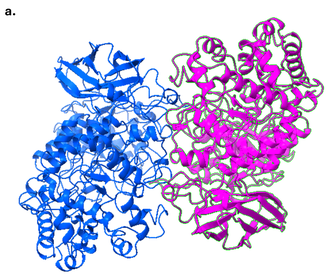

Figure 4: Computed Structural Model of Fsa15405Xyn dimer. a. This model was generated using the AlphaFold 2 plug-in on Chimera X. **b.** The predicted aligned error (PAE) plot associated with the CSM reflects the high confidence of the structure. The pTM value was 0.961. The ipTM value was 0.945.

SDS-PAGE confirmed the size of the recombinant Fsa15405Xyn enzyme as 82 kDa (**Figure 2**). Native-PAGE revealed an enzyme complex of ~200 kDa, suggesting a dimer (**Figure 3**). A CSM structure also predicted a dimer (**Figure 4**). Alignment of Fsa15405Xyn with a high-resolution crystal structure of a reference GH3, shows an aspartic acid at position 270 as the probable catalytic nucleophile and a glutamic acid at position 489 as the probable acid/base residue. Site-directed mutagenesis will be conducted in the future to determine whether those residues are required for catalysis. The temperature range for Fsa15405Xyn is 70 °C to 110 °C. The optimal temperature for Fsa15405Xyn is 90 °C (**Figure 5**). The pH range for Fsa15405Xyn is 4.5 to 9.5. The optimal pH is 4.5-7.5 (**Figure 6**). Fsa15405Xyn is thermostable up to 90 °C, but degrades completely within an hour at 100 °C (Figure 7). Based on a Lineweaver-Burk plot (**Figure 8**), Fsa15405Xyn has a V_{Max} of 285.7 μM/min, a K_M of 85.4 μM, and a K_{cat}/K_M of 0.398913 $\mu M^{-1}s^{-1}$. The K_M and K_{cat}/K_M of Fsa15405Xyn are a little lower compared to other GH3 family enzymes. The V_{max} is similar to other members of the family. Strong activity on 4-nitrophenyl-β-xylobioside and the production of xylose monomers from xylan (928.7 μM from a 1% solution of beechwood xylan) provides strong evidence that Fsa15405Xyn is an exo-β-1,4-xylanase.

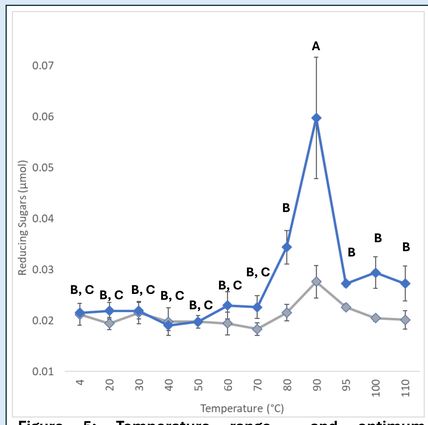

Figure 5: Temperature range and optimum. Fsa15405Xyn has a range of 70 °C – 110 °C compared to the empty vector control ($p \leq 0.05$ via unpaired t-tests) and an optimal temperature of 90°C. Error bars are based on standard deviation. Temperatures sharing a letter are not significantly different from each other based on an ANOVA with Tukey post-hoc analysis ($p \leq 0.05$).

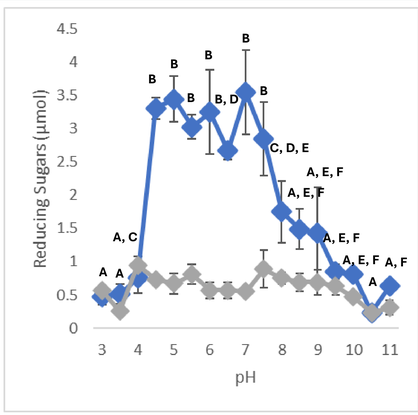

Figure 6: pH range and optimum. Fsa15405Xyn has a range of 4.5 – 9.5 compared to the empty vector control ($p \leq 0.05$ via unpaired t-tests) and an optimum pH of 4.5 to 7.5. Error bars are based on standard deviation. pH values sharing a letter are not significantly different from each other based on an ANOVA with Tukey post-hock analysis ($p \leq 0.05$).

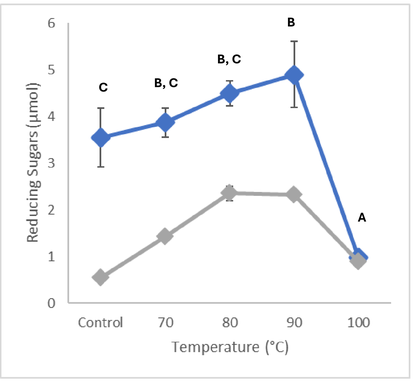

Figure 7: Thermostability. Fsa15405Xyn is stable up to 90°C. Error bars are based on standard deviation. Temperatures sharing a letter are not significantly different from each other based on an ANOVA with post-hoc Tukey HSD analysis ($p \leq 0.05$)

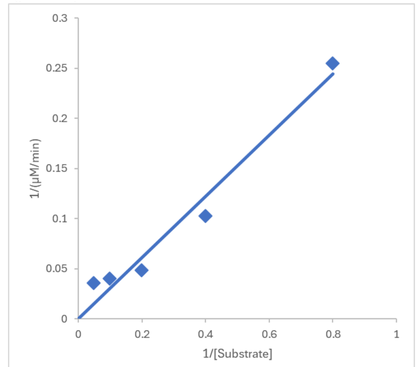

Figure 8: Lineweaver-Burk Plot. Fsa15405Xyn has a V_{Max} of 285.7 μM/min, a K_M of 85.4 μM, and a K_{cat}/K_M of 0.398913 $\mu M^{-1}s^{-1}$. The x-intercept was used to calculate K_M and the y-intercept was used to calculate V_{max} ($R2 = 0.9855$).

Conclusion

This study suggests that Fsa15405Xyn is an important component of an endo-xylanase/exo-xylanase system that allows for the degradation of xylan chains completely to xylose. Proteomics from *F. sacchari* in the presence of eight polysaccharides show that Fsa15405Xyn has low overall expression; however, in a mixed culture of *F. sacchari* with other microbes from GBS, Fsa15405Xyn is one of only 34 proteins expressed (Nou et al., 2024). Preliminary data from sugar metabolomics on flocs from GBS show that xylose-containing polysaccharides may be highly present in the sediment of GBS, where *F. sacchari* is found. This implies that xylose-containing polysaccharides may be highly available and labile in this environment for utilization as the substrate for this enzyme. Characterizing Fsa15405Xyn has contributed to a more complete picture of how *F. sacchari* lives in the extreme temperatures that occur in Great Boiling Spring, which is relevant to NASA's goal of understanding life in extreme environments.

Acknowledgments

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