Developing a Screening Method in Preparation for Directed Evolution of Cyanophycin Synthetase

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Introduction

Biopolymers offer biodegradable substitutes to many petroleum-derived plastics used today. Cyanophycin synthetase (CphA) is an attractive enzyme to target for novel polymer production due to its intriguing synthesis mechanism and the polymer's intriguing chemical structure[1]. The biopolymer cyanophycin is composed of a poly-L-aspartic acid backbone and multi-L-arginine side chains that are polymerized by CphA (Fig 1)[2]. This project outlines a possible screening method that later can be used in directed evolution to find mutant CphA variants which produce useful cyanophycin analogues.

Canavanine is an arginine analogue toxic to multiple strains of *E. coli.* CphA expressed in these *E. Coli* strains can incorporate canavanine into the polymer in place of arginine[1]. This process is thought to decrease system toxicity levels versus cells unable to sequester canavanine and thus provides a growth advantage. This screen compares the growth rates of CphA-expressing cells and wild-type (WT) cells in varying concentrations of canavanine.

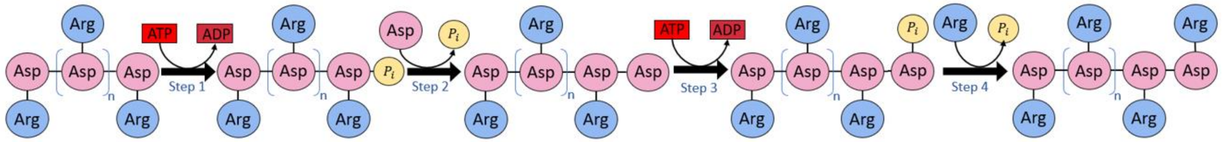


Fig 1: Proposed mechanism for the elongation of the cyanophycin polymer. ATP (red) is hydrolyzed by CphA to attach a phosphate moiety (yellow) onto the C-terminus side of the aspartic acid (pink) backbone (step 1). A free aspartic acid then replaces the phosphate moiety (step 2). ATP is again hydrolyzed to attach another phosphate moiety onto the sidechain of the newly attached aspartic acid (step 3). A free arginine (blue) then replaces the phosphate moiety (step 4).

Methods

BL21-DE3 *E. coli* strains were used for both CphA and WT cultures. A solution of a growth-media*, chloramphenicol, and IPTG was made for the stock solution. CphA and WT cells were then added separately and dispersed into individual wells. Concentrations of canavanine were then added into individual wells, maintaining a constant total volume. Growth rate of each well was monitored over 20 hours by a plate reader measuring optical density at 600 nm. The results for each triplet group were averaged and plotted for analysis.

***Solutions using TB, LB, and M9 media were all tested for project optimization.**
***A small amount of glycerol was included in the M9 media due to lack of other available nutrients**

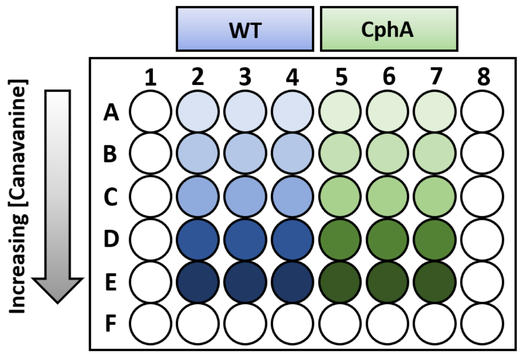
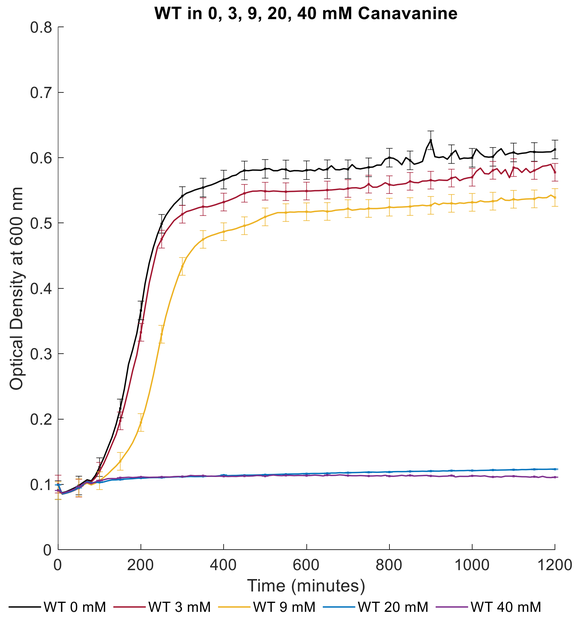


Fig 2: Top-down depiction of a 48 well plate. WT (blue) is in columns 2-4 & CphA (green) is in columns 5-7, both with canavanine concentrations increasing from A-E.

Findings – Canavanine Toxicity



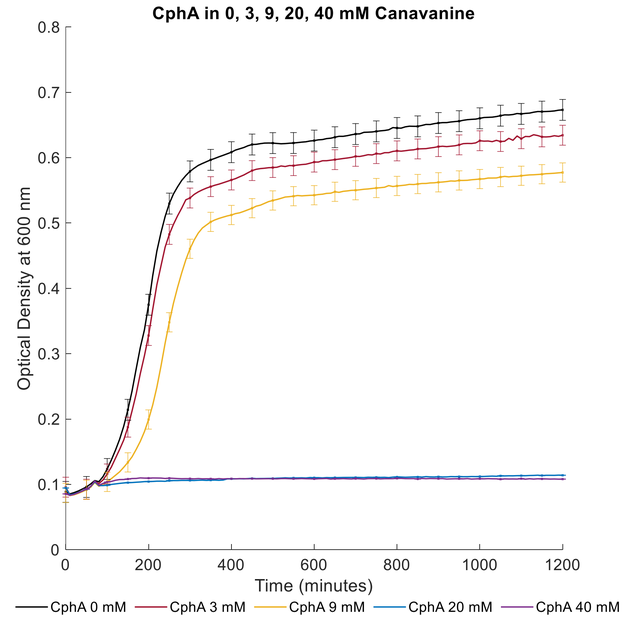


Fig 3: WT growth in LB stock over 20 hours with concentrations of 0 (black), 3 (red), 9 (yellow), 20 (blue), and 40 (purple) mM. Fig 4: CphA growth in LB stock over 20 hours with concentrations of 0, 3, 9, 20, and 40 mM. Increased canavanine concentration appears to decrease cell growth for both WT and CphA cells until death at 20 & 40 mM canavanine.

Discussion

The results indicates that canavanine is toxic to both WT and CphA cells, suggesting a failure to sequester canavanine into cyanophycin. We expect cells able to do this at a high rate would have a significant growth advantage over that of the wild-type. Further work involves the mutation of CphA by directed evolution, using this screen to find mutant variants with statistically significant growth advantages over the wild-type.

Acknowledgements

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[1] Aboulmagd E, Oppermann-Sanio FB, Steinbüchel A (May 2001). "Purification of Synechocystis sp. Strain PCC6308 cyanophycin synthetase and its characterization with respect to substrate and primer specificity". *Applied and Environmental Microbiology*. 67 (5): 2176–82. Doi:10.1128/AEM.67.5.2176-2182.2001. PMC 92852.
[2] J. Du, L. Li, and S. Zhou, "Microbial production of cyanophycin: From enzymes to biopolymers," *Biotechnology Advances*, vol. 37, no. 7, p. 107400, Nov. 2019.

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