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Assay Replicability in Enzyme Kinetics Across Laboratories

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Replicability is the foundation of research in any scientific discipline. Despite this fact, few studies address experimental variability within and across multiple institutions that operate under the same protocol. While consistency is usually well documented within the same lab, multi-institutional experiments may introduce new variables and, therefore, variability that may lead to inconsistent results. This study seeks to explore intra- and interinstitutional variability among enzyme catalytic efficiency values (K_M and k_{cat}/K_M) for the wild type of β-Glucosidase derived from *Paenibacillus polymyxa*. A standardized protocol for the assay was provided to all institutions that participated in the study. The analysis was conducted using data from 13 laboratories across the United States. The information was collected through the Design2Data CURE database. A total of 132 independent assays of β-Glucosidase were analyzed. Statistical analysis of k_{cat}/K_M , K_M, and T_{50} was conducted using SPSS, and Whisker Plots with a 90% confidence interval were generated for all parameters. Acceptance intervals for the parameters assayed of β-Glucosidase were determined; these could be used in all future experiments in the network as a reference by current and incoming laboratory members that incorporate into the network yearly. Ultimately, we aim to identify possible errors and misunderstandings in the protocol we use in the laboratories of the D2D network to improve assay replicability across institutions. High variability may be a concern when data is interpreted across institutions. This specific assay is used as a control for other, more complex assays in the CURE network and, therefore, the variability found can potentially lead to incorrect interpretation of results. It also highlights the high variability of a simple enzymatic assay when it is replicated in laboratories with different personnel and equipment, regardless of using the same standardized protocol.

Keywords: *replicability, beta-glucosidase, intra-laboratory, inter-laboratory*

Introduction

Design2Data (D2D) is a nationwide network of laboratories dedicated to exploring the triad of sequence, structure, and function. Originally established by four institutions (University of California (UC), Davis, Florida International University, Truckee Meadows Community College, and Hamline University), the network has since expanded, now encompassing ~40 universities and colleges. Through a C.U.R.E. (Course-based Undergraduate Research Experience) program, D2D-CURE aims to provide undergraduate students with an immersive research experience, as well as foundational research skills (Vater et al., 2021).

One of its notable projects involves the characterization of amino acid residues in β-Glucosidase B from *Paenibacillus polymyxa* via systematic mutations (Carlin et al., 2016). β-Glucosidases are a multi-organism enzyme family responsible for the hydrolysis of glycoside, oligosaccharides, and cellulose, cleaving the glycosidic bond and having glucose as an end-product (Cairns et al., 2010). The presence of glucose inhibits the chemical reaction (Singhania et al., 2012). They are present in all domains of life, although their starting substrate differs based on the specific function and organism of origin. Due to its widespread occurrence and enzymatic activity, this family is a subject of interest in various fields, such as agriculture, engineering, and medicine. In humans, decreased hydrolysis of glucocerebrosides is the cause of Gaucher's disease, as leukocyte β-Glucosidase has a lower enzymatic activity (Beutler et al., 1971). Some β-Glucosidases also have an application in oenology, as they are capable of hydrolyzing monoterpenes without altering the taste of wine and grapes (Rosi et al., 1994).

Within the D2D framework, students are guided through a three-phase journey centered around their β-Glucosidase enzyme. Students design their mutant variant, build it, and subsequently test its properties, comparing its catalytic and thermal parameters to the wild-type. This hands-on approach is further enriched by the integration of advanced software and online resources, including Foldit Standalone, Jalview, the D2D database, and BLAST (Vater et al., 2021).

FoldIt Standalone, a graphical interface evolved from the RoseTTA molecular prediction program, allows users to mutate known enzyme structures and it provides insights into molecular stability by generating an energy score, as an indicator for thermostability (Kleffner et al., 2017). However, like its counterparts—including AlphaFold2, RoseTTAFold, intFOLD7, and MULTIFold—FoldIt Standalone relies on computational algorithms to approximate these scores (Schutze et al., 2022; Outeiral et al., 2022). These computational strategies can occasionally be inaccurate. They might overlook the diverse ensemble of protein conformations and sometimes sidestep biophysical properties and the "two-state" processes inherent to protein folding (Outeiral et al., 2022).

The limitations of computational tools, such as FoldIt Standalone, validates the relevance of networks like Design2Data. D2D provides data that serve as a benchmark to challenge and validate the predictions made by such software (Vater et al., 2021). By introducing variant amino acids and analyzing the mutated enzyme's expression patterns—thermostability, catalytic efficiency, and substrate affinity—a comprehensive dataset can be created. This information is compared against the algorithmic predictions of FoldIt Standalone, particularly its energy score estimations and expression predictions. Discrepancies between experi-

mental outcomes and computational predictions are documented, offering invaluable insights to refine and enhance the underlying algorithms of the software.

Because of this quality of the D2D project and the evaluation of mutant products, it becomes of increased importance for universities to have a standardized protocol and produce baseline comparison wild-types. Since D2D is a multi-institutional network, all laboratories must run their own wild-types along their mutation assays per the network-wide procedures. The data from each laboratory is then uploaded to D2D for keeping records of all wild-types generated, which serves as a baseline to compare the new mutants (Huang et al., 2020). Consequently, the ability of laboratories to produce similar and consistent results becomes of paramount importance with this approach.

There are not too many studies that had explored this issue. At least a previous study found that even with standardized protocols, results tend to diverge in intra and inter-laboratory experiments (Pan et al., 2010), though the sources of error vary. Our paper aims to further investigate this discrepancy. The D2D nationwide network is in a unique position to contribute to understanding replicability within laboratories and variability across multiple institutions.

Methods and Procedures

For this study, a detailed set of sequential instructions for testing β-glucosidase was provided to thirteen institutions actively engaged in the Design2Data (D2D) data collection initiative, which includes universities across the United States. Along with the experiment protocol, the institutions were also provided with a list of distributors from which they could purchase reagents, materials, and cell cultures.

Each laboratory was tasked with running a wild-type assay to act as a control along with their selected mutants. This control is essential for comparative analysis and ensuring the integrity of the experimental protocol and the investigated mutations.

1. Wild-Type Expression

In each laboratory section, four wild-type replicates were expressed as a baseline for comparison. The β-glucosidase wild-type plasmid was bioengineered at our partner institution, UC Davis, and then provided to all other institutions. Two microliters of β-glucosidase wild-type plasmid were transformed into *E. coli* DH5α chemically competent cells. Transformation was achieved through multiple rounds of ice incubation followed by a heat shock. Subsequently, the cells were plated on LB/Kan+ (50 μg/mL) Petri dishes and allowed to incubate for 16 hours at 37°C. After bacterial colonies grew, one was selected from each wild-type Petri dish and inoculated into LB media containing kanamycin. Cells were incubated overnight at 37°C in a shaker at 255 RPM.

The next morning, bacteria were collected by centrifugation and plasmid DNA was isolated using a QIAprep Spin Miniprep Kit. β-glucosidase plasmid was Sanger sequenced at Eurofins Genomics (Louisville, KY) to confirm the plasmid sequence.

In order to express β-glucosidase, 2 μL of β-glucosidase plasmid were transformed into *E. coli* BL21(DE3)

chemically competent cells. Cells from this transformation were plated onto petri dishes with kanamycin (50 µg/mL). The bacterial cultures were incubated overnight at 37°C. The next day, a single colony was selected and cultured in LB media with Kanamycin for 16 hours at 37°C and shaking at 255 RPM. After incubation, IPTG was added to the LB media to induce protein expression for 16 hours at 20°C, shaking at 255 RPM. The following day, cells were collected by centrifugation and stored at -20°C until protein extraction and purification were performed.

β-glucosidase was previously tagged with a poly-histidine tag. This distinctive molecular marker enabled its purification using immobilized metal ion affinity chromatography using Ni-NTA. The purified β-glucosidase underwent SDS-PAGE analysis, a technique employed alongside other BgIB variants, to check for the quality of the protein purification process.

2. Kinetic and Thermostability Assay

To assess the enzymatic activity of BgIB, 4-nitrophenyl-β-D-glucopyranoside was used as substrate. Upon enzymatic activity and hydrolysis of the bond between the nitrophenyl group and the sugar moiety, a yellow color is released. The intensity of the color yellow was used to determine the dilution factor used for the thermo and kinetic assays,

For the kinetic assay, a 96-well plate was set up with varying substrate stock concentrations through serial dilution. Initially, 150 µL of substrate stock was added to row A using a single-channel pipette. For rows B through H, 100 µL of elution buffer was added. Then, 50 µL of substrate stock was serially transferred from row A to B, B to C, and so on using a multi-channel pipette. For the protein plate in the kinetic assay, 25 µL of the diluted wild-type was added to the first three columns. Then, 75 µL from the substrate plate was transferred into the protein plate, initiating the enzymatic assay.

For the thermal assay, 14 µL of BgIB protein was combined with 1386 µL of the elution buffer in a small plastic tube. From this mixture, aliquots of 150 µL were dispensed into an 8-PCR tube strip, each corresponding to a distinct temperature. Each plastic tube was incubated for 30 minutes in a thermal cycler with a gradient from 50°C to 30°C. Subsequently, the substrate plate was readied by transferring 75 µL of a diluted substrate solution into all 96 wells. An enzyme plate was then arranged using 50 µL triplicates for each PCR tube. Finally, 25 µL from the prep plate was transferred into the enzyme plate for each well to start the enzymatic reaction.

A spectrometer operating at a wavelength of 420 nm was used to detect the amount of product being released in the reaction. Subsequently, the obtained data points were uploaded into the Design2Data (D2D) repository website. These data were then used for graphical representations of the data and determination of the thermal and kinetic parameters of the enzyme.

Three parameters were calculated were K_M, k_{cat}/K_M, and T_{50}. The term K_M refers to the Michaelis constant, a substrate concentration value that corresponds to the reaction velocity that is half of the maximum velocity, V_{max}. Similarly, k_{cat}/K_M refers to the specificity constant, a value that helps assess the catalytic efficiency of the enzymes. Lastly, T_{50} indicates the temperature at which 50% of the enzyme is still functional

and is used as an indicator of thermal stability.

3. Interpretation of nationwide results

Data was collected from the Design2Data (D2D) online database (https://www.d2dcure.com/). The data was separated into two categories: curated and non-curated. Curated data reflected an in-depth analysis performed by laboratory supervisors to assess for any possible errors of input or invalid data-points, whereas non-curated data was raw data. All curated wild-type data points were obtained from the institutions of the network and analyzed through a Kruskal-Wallis test via the statistical software Statistical Product and Service Solutions (SPSS). Curated and non-curated data points were then used to construct Whisker Plots with 90% confidence range. Finally, a comprehensive table was produced showcasing medians and intervals for each tested parameter: K_M, k_{cat}/K_M, and T_{50}.

Results

An Independent-Samples Kruskal-Wallis Test was conducted using the SPSS computational tool to assess the validity of the reported data. The test was conducted under the assumption of a null hypothesis, positing that the data points were uniformly distributed across institutions within each category. For the purpose of evaluating reliable data, the analysis was done on the curated dataset—a collection of experimental values that the lab supervisor of each institution has reviewed. The confidence interval was set at 95%, and the significance level was chosen as 0.050. The analysis was performed on three distinct variables: K_M, k_{cat}/K_M, and T_{50}.

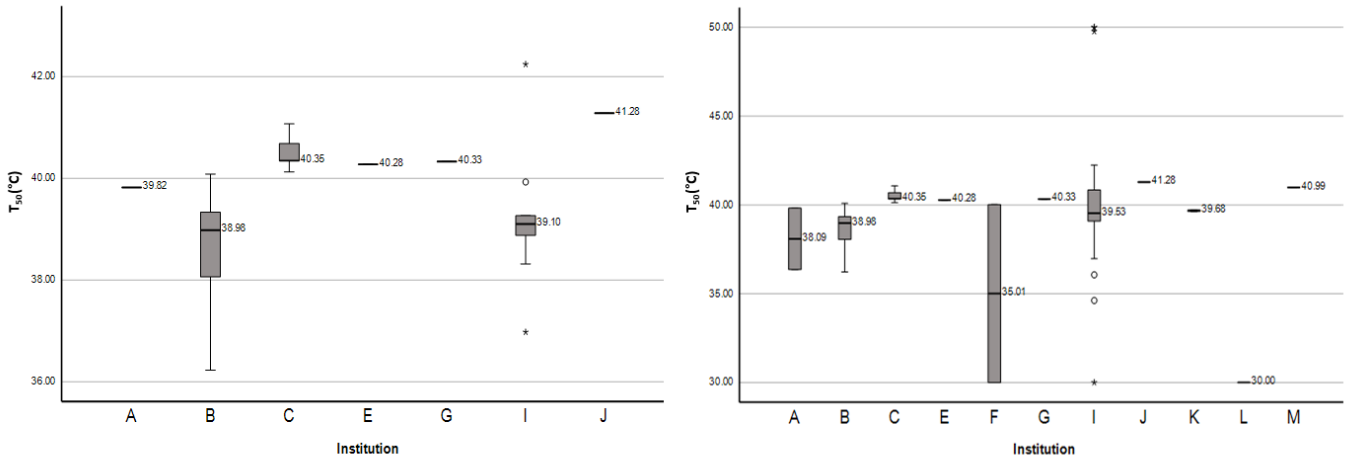
For the K_M parameter, the computed significance value was 0.573, based on a total sample size (n) of 79. The Kruskal-Wallis test statistic yielded a value of 7.620a, corresponding to a degree of freedom of 0. The statistical analysis resulted in the retention of the null hypothesis. This indicates that no significant differences were observed in the distribution of K_M values across the various institutions.

In the case of k_{cat}/K_M, the computed significance value was found to be less than 0.001, considering a total sample size (n) of 79. The Kruskal-Wallis test statistic was calculated as 37.474a, accompanied by a degree of freedom of 9. As a result of this analysis, the null hypothesis was rejected. This implies the existence of statistically significant differences in the distribution of k_{cat}/K_M values across the studied institutions.

The T_{50} variable yielded a significance value of 0.002, derived from a total sample size (n) of 40. The Kruskal-Wallis test statistic was calculated to be 20.081a, with a degree of freedom of 6. Consequently, the null hypothesis was rejected. This outcome indicates the presence of statistically significant differences in the distribution of T_{50} values among the institutions.

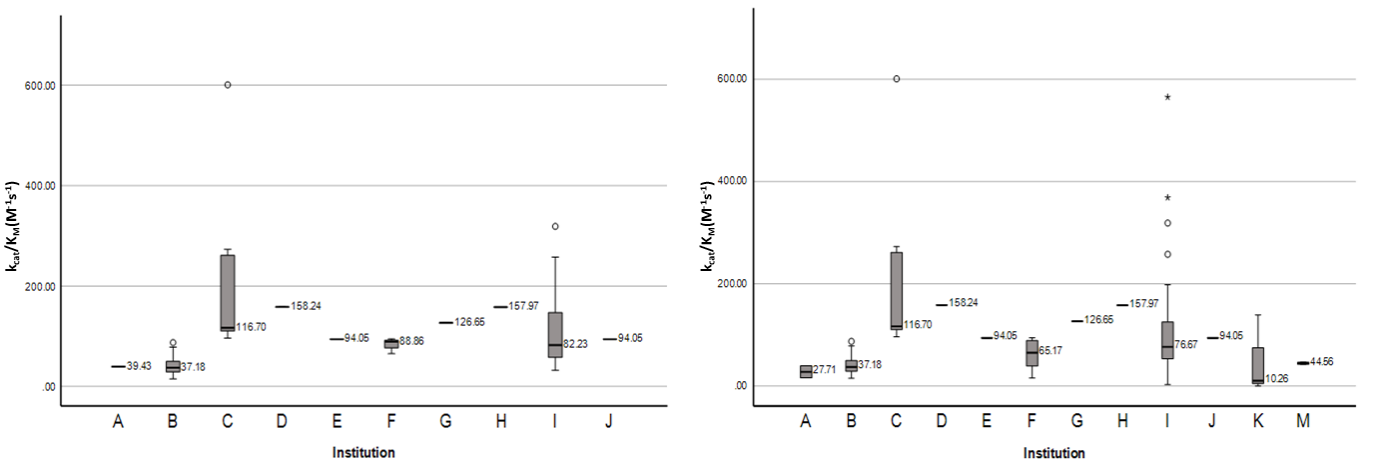
Furthermore, for a better comparison of data in between institutions, 90% confidence interval Whisker Box plots were created for curated, as well as non-curated datasets. This allowed for a side-by-side comparison.

Figure 1.0



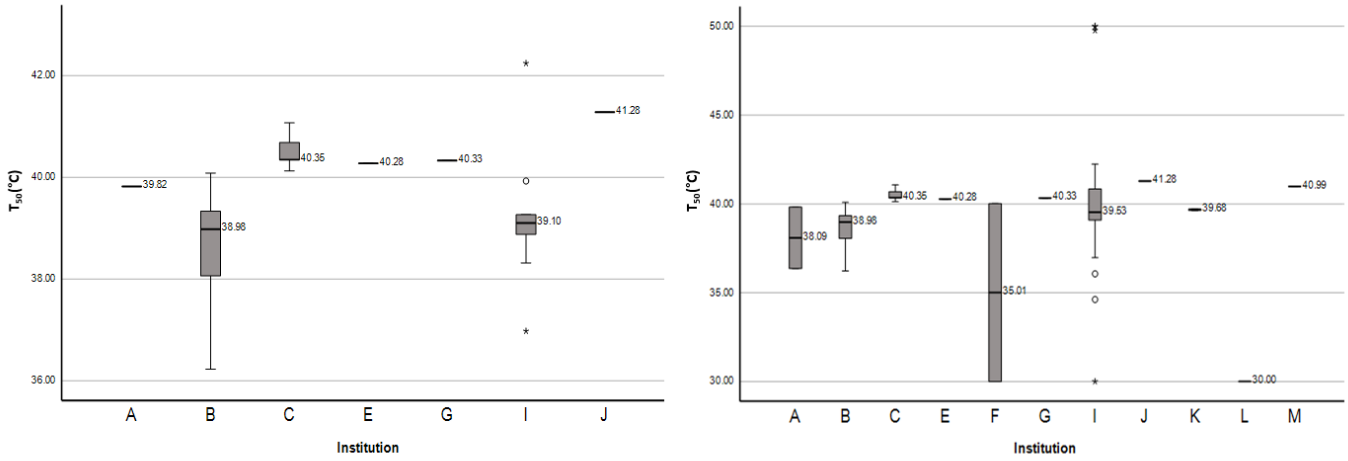
Note: Whisker Plots (90% confidence) for K_M values from participating institutions. The side-by-side comparison illustrates the difference between curated (left) and non-curated data (right). Participating institutions are labeled A-M. Circles represent data points outside of the 90% confidence interval, and asterisks depict outliers identified by SPSS as deviating significantly from the dataset's norm.

Figure 2.0



Note: Whisker plot (90% confidence) for k_{cat}/K_M from participating institutions. The side-by-side comparison illustrates the difference between curated (left) and non-curated data (right). Participating institutions are labeled A-K. Circles represent data points outside of the 90% confidence interval, and asterisks depict outliers identified by SPSS as deviating significantly from the dataset's norm. Institution L was excluded from the graph to ensure readability; however, it was included in the statistical analysis.

Figure 3.0



Note: Whisker plots (90% confidence) for T_{50} values participating institutions. The side-by-side comparison illustrates the difference between curated (left) and non-curated data (right). Participating institutions are labeled A-K. Circles represent data points outside of the 90% confidence interval, and asterisks depict outliers identified by SPSS as deviating significantly from the dataset's norm. Some institutions did not report T_{50} values.

Table 1

	Median K_M (mM)	Median k_{cat}/K_M (M-1s-1)	Median T_{50}	Interval K_M (mM)	Interval k_{cat}/K_M (M-1s-1)	Interval T_{50}
Curated	**4.2**[2]	**94.29**	**40.13**	**2.79-6.5**[0]	**53.69-197.9**[6]	**38.77-40.8**[3]
Non-Curated	**4.89**	**63.14**	**39.69**	**2.42-8.3**[8]	**18.45-129.3**[5]	**37.26-45.2**[6]

Note: This table presents the calculated wild-type median values along with their corresponding 90% confidence intervals for both curated and noncurated datasets across all participating institutions. Notably, one specific data point originating from University A was deliberately excluded from the calculations due to being an in-put error, not an experimental one.

The wild-type median K_M value for the curated dataset was 4.22 mM, and the 90% confidence interval range of 2.79-6.50 mM. The median K_M value for the non-curated dataset was 4.89 mM, and the 90% confidence interval range of 2.42-8.38 mM. This analysis was conducted on a comprehensive sample pool consisting of 136 data points. Notably, the reported findings vividly demonstrated substantial variability, as

depicted in **Figure 1**.

The wild-type median value for the k_{cat}/K_M for curated data was 94.29 M-1s-1, and the 90% confidence interval was 53.69-197.96 M-1s-1. The recorded non-curated data reported a median of 63.14 with a 90% confidence interval of 18.45-129.35 M-1s-1. Based on a total sample size of 136, this indicated high levels of variability between institutions, as shown in **Figure 2**.

Among the 13 participating institutions, T_{50} values were reported based on data from 11 of them for the non-curated dataset, and 7 of them for the curated dataset. For the curated data subset, the median T_{50} value was determined to be 40.13 °C, with a 90% confidence interval spanning 38.77-40.83°C. Conversely, the non-curated data subset exhibited a median T_{50} value of 40.45°C, accompanied by a 90% confidence interval ranging from 37.26-45.26°C.

Discussion

The analysis of both curated and non-curated datasets extracted from the Design2Data database reveals a notable degree of intra-institutional variability in the obtained results. This observation underscores the complex nature of enzyme kinetics experiments, where numerous factors such as experimental conditions, equipment calibration, and sample preparation can introduce variations. The identification of outliers through statistical methods, facilitated by SPSS, provides a mechanism to pinpoint and investigate discrepancies in reported data. These outliers can now be subjected to closer scrutiny within their respective laboratories, enabling the identification of potential sources of error and the refinement of experimental procedures.

The independent-sample Kruskal-Wallis test shows that k_{cat}/K_M and T_{50} contained a higher degree of variability. The measurement for K_M, despite showing a wide range of values, is more consistent across institutions. This suggests that the variability for k_{cat}/K_M is more closely related to the value of the turnover number, k_{cat}.

Our analysis shows that some institutions deviate from the reported median values of their counterparts. Institution L reports k_{cat}/K_M values that are 1000 times higher than the median values reported by other universities. Institution C reported k_{cat}/K_M values that range from 2 to 4 times higher than the established norm. Possible explanations for these discrepancies include variations in the number of wild-type contributions, differing enzyme dilution factors, protein quantification, or human error in data recording.

Interestingly, some institutions did not include a report for the T_{50} values or the quantity of wild-type enzymes expressed in their assays. The absence of these data hinders a comprehensive analysis and comparison of experimental outcomes. It is important to have a large and comprehensive dataset for a complete and holistic analysis of enzymatic kinetics results and to foster reproducibility across research institutions.

A significant finding emerges from the analysis of curated data, which displays a narrower range for the majority of parameters studied. This emphasizes the value of meticulous data curation by supervisors in maintaining the quality and relevance of the information stored in the D2D database. The curated data's reduced variability further emphasizes the importance of standardizing experimental procedures and ad-

hering to reporting guidelines, ultimately enhancing the reliability of enzymatic kinetics research findings.

The provision of 90% confidence intervals for K_M, k_{cat}/K_M and T_{50} parameters serves as a valuable resource for guiding new institutions in designing and executing their enzyme kinetics experiments. These intervals offer a robust framework for assessing the precision and reliability of reported results. These intervals are provided in hopes of identifying and eliminating experimental errors prior to their upload to the network by comparing outcomes to expected values. Additionally, they can be used by non-network affiliates in investigations of β-Glucosidases.

In conclusion, this study's multifaceted analysis of the D2D database highlights various aspects of enzyme kinetics research. The provision of confidence intervals aids in guiding new institutions, the observation of intra-institutional variability prompts introspection and improvement, and the identification of deviations and outliers helps identify potential problems in the assays and the final quality of the data reported. Furthermore, the absence of key data points and the narrower range exhibited by curated data collectively emphasize the relevance of meticulous data curation and adherence to protocols. As the field of enzymatic kinetics advances, these insights underline the need to refine methodologies and bolster the quality of research data reporting.

References

Beutler, E., Kuhl, W., Trinidad, F., Teplitz, R., & Nadler, H. (1971) Beta-glucosidase activity in fibroblasts from homozygotes and heterozygotes for Gaucher's disease. *Am J Hum Genet, 23*(1), 62-66. PMID: 5581982; PMCID: PMC1706631.

Carlin, D. A., Caster, R. W., Wang, X., Betzenderfer, S. A., Chen, C. X., Duong, V. M., Ryklansky, C. V., Alpekin, A., Beaumont, N., Kapoor, H., Kim, N., Mohabbot, H., Pang, B., Teel, R., Whithaus, L., Tagkopoulos, I., & Siegel, J. B. (2016). Kinetic Characterization of 100 Glycoside Hydrolase Mutants Enables the Discovery of Structural Features Correlated with Kinetic Constants. *PLoS One, 11*(1). 1-14. https://doi.org/10.1371/journal.pone.0147596

Chakravarty, D., Porter, L. L. (2022). AlphaFold2 fails to predict protein fold switching. *Protein Sci, 31*(6). 1-11. https://doi.org/10.1002/pro.4353

Huang, P., Chu, S. K. S., Frizzo, H. N., Connolly, M. P., Caster, R. W., & Siegel, J. B. (2020). Evaluating Protein Engineering Thermostability Prediction Tools Using an Independently Generated Dataset. *ACS Omega, 5*(12). 6487-6493. https://doi.org/10.1021/acsomega.9b04105 Miller, J. L. (2021). Deep learning opens up protein science's next frontiers. *Physics Today, 74* (10). 14–17. https://doi.org/10.1063/PT.3.4850

Ketudat Cairns, J. R., & Esen, A. (2010). [Beta]-glucosidases. *Cellular and Molecular Life Sciences, 67*(20), 3389-405. https://doi.org/10.1007/s00018-010-0399-2

Kleffner, R., Flatten, J., Leaver-Fay, A., Baker, D., Siegel, J. B., Khatib, F., & Cooper, S. (2017). Foldit Standalone: a video game-derived protein structure manipulation interface using Rosetta.

Bioinformatics, 33(17). 2765–2767. https://doi.org/10.1093/bioinformatics/btx283

Mirdita, M., Schütze, K., Moriwaki, Y., Heo, L., Ovchinnikov, S., & Steinegger, M. (2022). ColabFold: making protein folding accessible to all. *Nature Methods* 19, 679–682 https://doi.org/10.1038/s41592-022-01488-1

Outeiral, C., Nissely, D. A., & Deane, C. M. (2022) Current structure predictors are not learning the physics of protein folding. *Bioinformatics, 38*(7).1881–1887. https://doi.org/10.1093/bioinformatics/btab881

Pan, Y., Bodrossy, L., Frenzel, P., Hestnes, A. G., Krause, S., Lüke, C., Meima-Franke, M., Siljanen, H., Svenning, M. M., Bodelier, P. L. (2010). Impacts of inter- and intralaboratory variations on the reproducibility of microbial community analyses. *Applied Environmental Microbiology, 76*(22). 7451-7458. https://doi.org/10.1128/AEM.01595-10

Rosi, I., Vinella, M., & Domizio, P. (1994). Characterization of beta-glucosidase activity in yeasts of oenological origin. *J Appl Bacteriol, 77*(5). 519-27. https://doi.org/10.1111/j.13652672.1994.tb04396.x.

Singhania, R. R., Patel, A. K., Sukumaran, R. K., Larroche, C., & Pandey, A. (2012). Role, and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. *Bioresource Technology,* 127. 500-507. https://doi.org/10.1016/j.biortech.2012.09.012.

Vater, A., Mayoral, J., Nunez-Castilla, J., Labonte, J. W., Briggs, L. A., Gray, J. J., Makarevitch, I., Rumjahn, S. M., & Siegel, J. B. (2021). Development of a Broadly Accessible, Computationally Guided Biochemistry Course-Based Undergraduate Research Experience. *Journal of Chemical Education, 98*(2), 400-409. https://doi.org/10.1021/acs.jchemed.0c01073