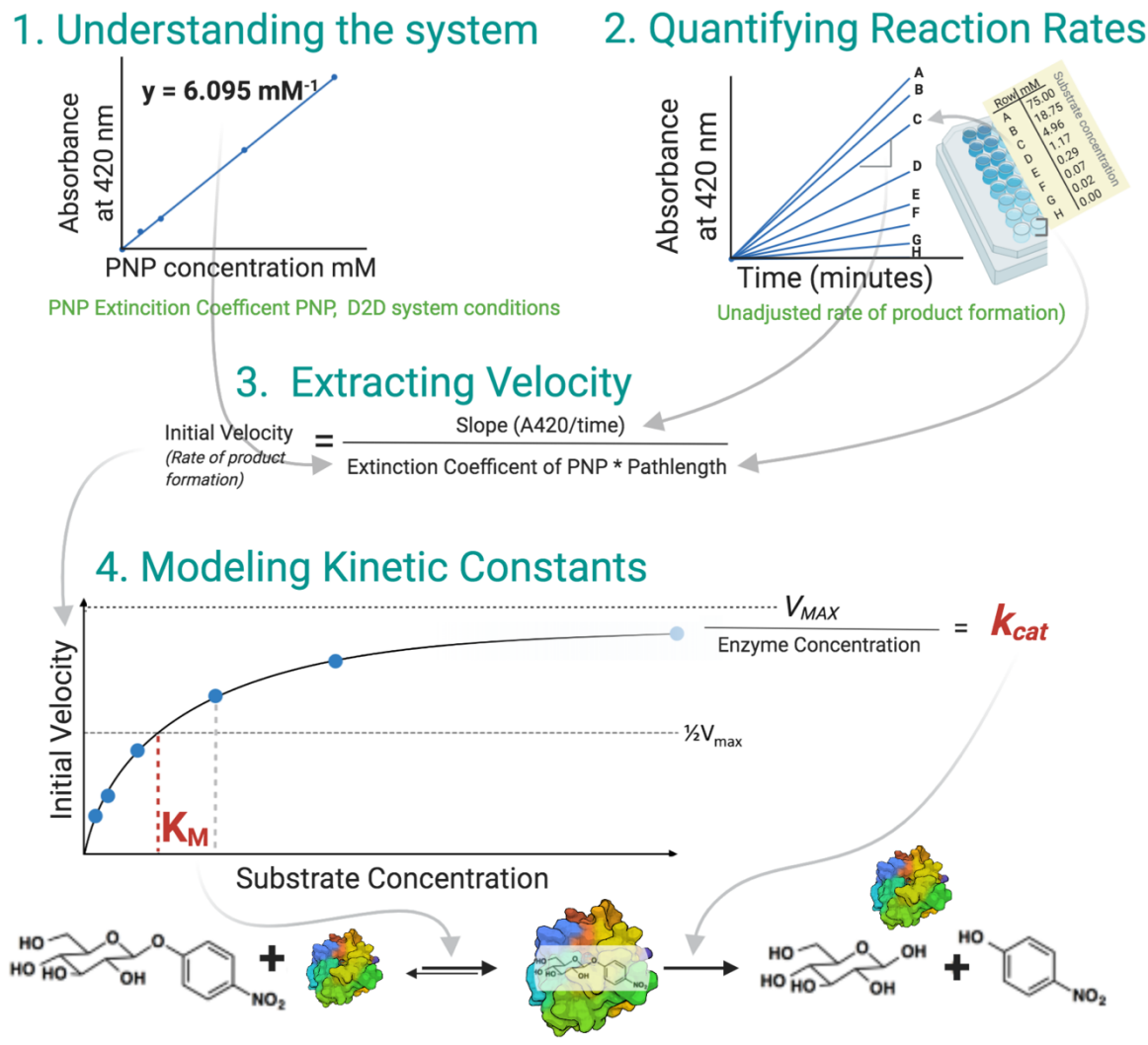
Guide to interpreting colorimetric assay data with enzyme kinetics

Finally, after a long semester of hard work designing, building and testing your mutant, you will be able to characterize its enzymatic activity! The study of the 3-D structure of the protein you did on β-glucosidase B at the beginning of the semester, together with the mechanistic approach you took to determine the functional effects of specific mutations, will provide novel information about the relationship between of individual amino acids in enzyme structure and enzyme mechanism. This is vital information we currently lack for 99% of the enzyme we know! This guide refresh your background knowledge about the system and the will dive into the topics: Quantifying Reaction Rate, Data Quality Control, Extracting Velocity, and Modeling Kinetic Constants.



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1. Understanding the system

Kinetics is all about measuring rates. In chemistry, a reaction rate is defined as change in concentration over change in time. In enzyme kinetics, things are no different; we are concerned with the change in concentration of products or substrates as an enzyme reaction progresses.

Since we cannot see the actual molecules reacting inside an enzyme, we must observe them indirectly, using an enzyme assay. This assay often involves monitoring the change in color as a reaction progresses.

In this summary, we explain how changes in the color of our enzymatic reaction are used to measure the catalytic efficiency of our mutations. One of the most important features of enzymes is their catalytic activity, which consists of two features: (1) how readily the enzyme binds to the substrate, and (2) how quickly the enzyme is able to turn over reactions.

1.1) β-glucosidase enzyme reaction

For starters, recall that β-glucosidase B hydrolyzes sugars. We can give this enzyme an artificial substrate – in this case 4-nitrophenyl-B-D-glucopyranoside – which is essentially a sugar attached to a nitrophenol. This substrate appears clear in solution but when it is broken down (by our enzyme) one of the products will be P-nitrophenol (PNP), which in solutions with pH greater than or equal to 7.15 will be deprotonated and appear yellow.

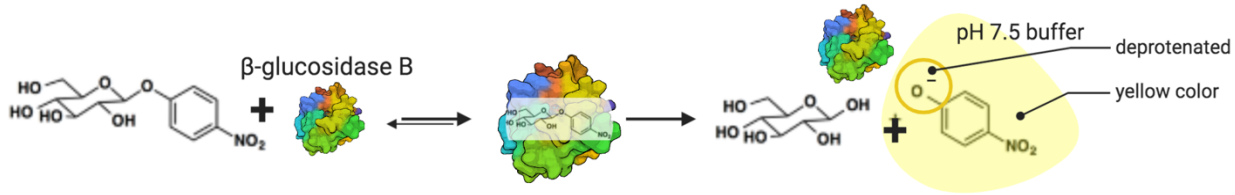


Figure 1. β-glucosidase B hydrolysis reaction used for evaluating enzyme kinetics and showing the conditions that are part of our experimental system.

1.2) Visualizing the reaction

In our case, we are observing the release of p-nitrophenol (PNP) from the substrate as the enzyme performs its reaction, the initial rate, or *initial velocity (V_o)*. Essentially, we can watch the concentration of PNP increase over time. In other words: the more yellow, the more product has formed. We can quantify the change in yellowness using a spectrophotometer.

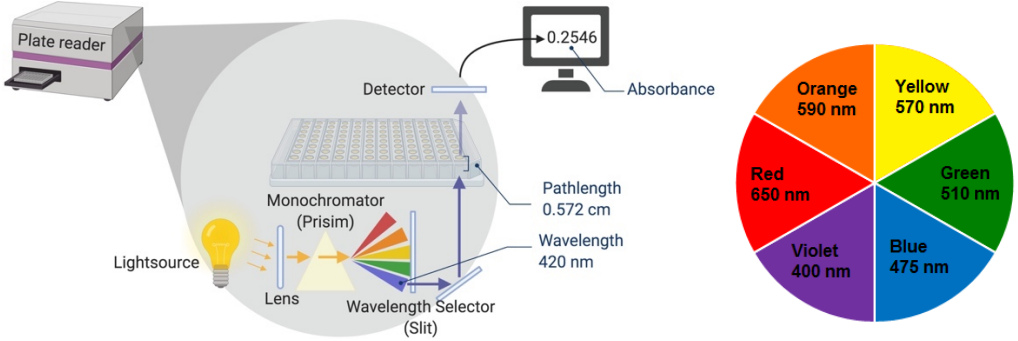


Figure 2. We use the light wavelength 420nm to measure yellowness. This is because our PNP solution strongly absorbs purple light and reflects yellow light, which we visualize as the color of the solution. Notice that light of 420 nm wavelength is across from yellow and will be optimal for measuring absorbance of PNP.

1.3) Understanding absorbance

When we measure absorbance, we are looking at the difference between the amount of light that was sent into the sample and the amount of light that comes out the other side. Some substances absorb light (at a particular wavelength) more readily than others. We need to factor this into our calculations, and we call it the extinction coefficient (ε). We will use this factor to normalize the raw absorbance values the spectrophotometer plate reader generates.

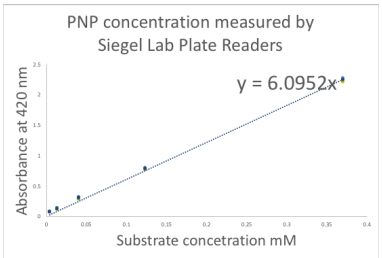


Figure 3. We calculated the absorbance/substrate relationship as 6.0952 mM^{-1} at 420 through a standard curve assay with serial dilutions from 0 to 10 micromolar PNP using a MulitSkan Sky plate reader. The linear section of the curve, used for the calculation, was determined from PNP concentrations ranging from 0.0046 to 0.3704 mM.

To complete our extinction coefficient calculation, we include the pathlength of the sample. In our case – the 96-well half area flat bottom plate, Corning cat. # 3884 – filled with a volume of 100 µL has a pathlength of 0.572 cm (Figure 2; Corning, 2020). **Thus, the extinction coefficient under our experimental conditions = 10.66 $mM^{-1}cm^{-1}$.** This is somewhat different than previously published values in similar experiments but not outside a factor of two and likely the variation is due to differences in pH and standard curve assay wavelength between our system (A420) and the published one (A400) (Bowers et al, 1980).

1.4) Rate of product formation in relationship to substrate concentration

When we vary the amount of substrate in our experimental system, we can think about what's happening at the molecular level in two different, key scenarios to help us understand our enzyme's catalytic efficiency (Figure 4). We have set up our experiment with the following substrate concentrations (Table 1).

Table 1. Substrate concentrations used in the experimental system for Design-2-Data workflow.

Row	Substrate concentration mM
A	75
B	18.75
C	4.69
D	1.17
E	0.29
F	0.07
G	0.02
H	0

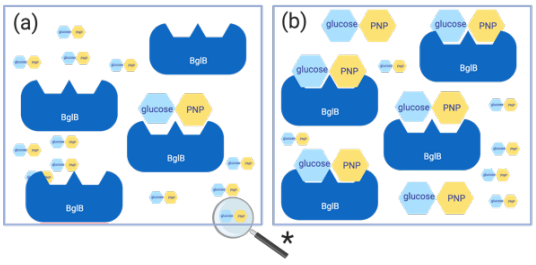


Figure 4. Cartoon of different enzyme-to-substrate concentration experimental scenarios: (a) Scenario #1: "*low*" substrate concentration relative to enzyme concentration. (b) Scenario #2: high substrate concentration. *Note: It is essential to realize that there is always excess substrate in this experimental system, even in the "*low*" substrate concentrations.

1.5) Approximating binding affinity

Scenario #1 (Figure 4. a): Less substrate available. When there is little substrate available, the limiting factor for product formation is determined mostly by how readily the substrate binds to the active site of the enzyme. If the substrate is easily able to find the active site, more product will be converted in contrast to an enzyme where the binding affinity is lower. You can imagine that if the active site is very "tight" or somewhat blocked this would lower the **binding affinity, which we approximate with a value we call K_M.** This summary is arguably a gross oversimplification; for a more complete and correct description, review Berg, Tymoczko Stryer, 2002 (linked in References).

1.6) Observing turnover rate

Scenario #2 (Figure 4. b): More substrate than enzyme.
Here you can imagine that all of the enzyme is saturated with substrate and the thing that will vary product formation under this condition is how quickly and enzyme can "boot out" the product. You can imagine that if this is a multistep reaction, with lots of moving parts, it will be slower to expel the product than a reaction that only has a step or two, which can occur more rapidly. **We call this turnover rate k_{cat}.**

2. Quantifying reaction rate

Recall the way we set up our plate with substrate concentration ranging from concentrated (topmost rows) to very dilute (bottommost rows). Table 2 shows an example of the data produced. The values in the middle of the table represent absorbance as calculated by the spectrophotometer.

Table 2. Example of absorbance value of each well – assigned to a substrate concentration – calculated at each timepoint. There are only 4 timepoints shown here for simplicity. The assay we run will calculate absorbance at 15 time points of one-minute intervals. Note, the absorbance data that comes off the reader is initially unitless.

	[S]	Time 1	Time 2	Time 3	Time 4
Well A1	1.0	1.0	2.2	3.1	4.1
Well B1	0.67	0.8	1.3	1.8	2.0
Well C1	0.33	0.1	0.4	0.6	0.9

We can simplify this data by representing it as a rate of absorbance over time. Remember that absorbance represents the amount of product in the solution. Since absorbance, being the logarithm of the ratio of light transmitted and light received, has no units[1], using the below equation provides a rate in units of mM PNP/min, if the provided slope is in inverse minutes. We select the rate data in the steepest, linear phase of the reaction for our analysis (Figure 5). A critical note about the Mulitskan Sky plate reader and Skanit software: you will notice that when setting up the assay, the software will give you options for the unit of time for calculating the rate of product formation. You should select the option: "*Kinetic rate unit: 1/min*".

[1] Many scientists wrongly use a synonym for absorbance (A), optical density (OD), as if it were a unit, for example recording an absorbance of "0.500 OD" instead of simply 0.500. This problem is enhanced by the fact that many instruments provide values in mOD, that is "milliOD", so a measurement reported by such a machine with an absorbance of "500 mOD" is in fact an absorbance of 0.500.

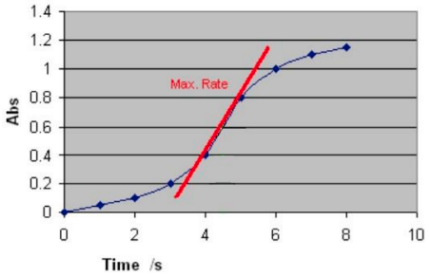


Figure 15. Determining the maximum rate with window value 2

Figure 5. Maximum rate determined from linear portion of reaction over time, our rate is determined over 3-point window (Thermo, 2015).

Table 3. Example *A420 min*$^{-1}$ data generated by MulitSkan Sky plate reader and software showing triplicate data for wild type example. The full data table produced by the Skanit software will included all 12 columns. This is the example we'll be using throughout the document. Absorbance is unitless but pathlength adjusted so carries units: cm min^{-1}.

Rate	1	2	3
A	1.38E-01	1.40E-01	1.38E-01
B	1.12E-01	1.08E-01	1.10E-01
C	7.36E-02	7.08E-02	7.04E-02
D	2.52E-02	2.30E-02	2.50E-02
E	6.69E-03	5.85E-03	6.49E-03
F	1.89E-03	1.47E-03	1.57E-03
G	6.99E-04	5.41E-04	5.22E-04
H	0	0	2.30E-05

2.1) Dealing with triplicate data and error checking

Here, you should review your data to verify that the rates for the triplicates look fairly uniform. If there are obvious outliers, you might consider removing them. Because the triplicates are sets of three adjunct columns, i.e. columns 1, 2, and 3 comprise the Wild Type plate control, you should move forward by taking the averages of each plate.

3. Extracting velocity

Our kinetic assay is designed to collect data from low to high substrate concentrations so that we can observe the rate of product formation under those varied conditions.

3.1) Translating slope – or rate – of product formation into initial velocity of the reaction

We need to make a couple small adjustments to translate the calculated slope values (Table 2) into more a standardized metric. Remember the extinction coefficient we mentioned when we described how absorbance is calculated? We need to normalize our *rate of product formation* with that extinction coefficient of the product (PNP) and pathlength of the sample to be able to compare our results to those of other experiments.

We can think of this as an application of the Beer–Lambert law, which is $A = \varepsilon lc$, where ε is the extinction coefficient in mM^{-1} cm^{-1}, l is the path length in cm, and c is the concentration in mM. Rearranging the Beer–Lambert law ([PNP] = $c_{PNP} = A_{420\ nm}/\varepsilon_{PNP}l$), we can calculate the rate of PNP release from the steepest slope[1] . For example, if we measure an initial steepest slope of $A_{420\ nm}$ of 0.14 min^{-1}.

$$v = \frac{slope}{\epsilon_{\mathrm{PNP}} \cdot l} = \frac{0.140\ \mathrm{min}^{-1}}{(10{,}660\ \mathrm{M}^{-1}\cancel{\mathrm{cm}^{-1}})(0.572\ \cancel{\mathrm{cm}})} = 2.30 \times 10^{-5}\ \mathrm{M/min}$$

Once normalized by the extinction coefficient of the product and the absorbance pathlength in our experimental system, we call this the *initial velocity (V_o)* of product formation.

3.2) Calculating the enzyme concentration in our experiment

Beyond including the product-related factors in this system, we also need to include the concentration of enzyme in the reaction to accurately compare our data to the kinetic results of other enzyme experiments. To start this process, we need to know the (1) extinction coefficient for our enzyme (as opposed to the extinction coefficient of PNP), (2) the enzyme dilution factors in our assay, and (3) the estimated protein concentration for the enzyme as reported in the A280 assay.

Table 4. Example values and system constants for enzyme calculation.

Variable	Value	Units	Notes
$A_{280\ nm}$	1.354	NA	This is an example, not a constant for the system
Dilution factor	100	X	Enzyme solutions are diluted based on a crude visual-eye-test for activity. 100 dilution value used in this example
ϵ_{BglB}	113,330	$M^{-1}cm^{-1}$	Calculated by method described here.
l	1	cm	Pathlength, we assume 1 cm based on A_{280} assay analysis software readout
$V_{E\ added}/V_{total}$	0.25	L/L	This is the ratio of diluted protein (V_E) added to the working substrate and enzyme solution (V_{total})

Input: Yield / $A_{280\ nm}$: 1.354 **Dilution Factor: 100×**

$$[\mathrm{E}]_{\mathrm{stock}} = \frac{A_{280\,nm}}{\epsilon_{\mathrm{BglB}} l} = \frac{1.354}{(113{,}330\ \mathrm{M}^{-1}\cancel{\mathrm{cm}^{-1}})(1\ \cancel{\mathrm{cm}})} = 1.195 \times 10^{-5}\ \mathrm{M}$$

(Note: the $A_{280\ nm}$ reading is preadjusted by the instrument for a path length of 1 cm.)

$$[\mathrm{E}]_{\mathrm{diluted}} = \frac{[\mathrm{E}]_{\mathrm{stock}}}{dilution\ factor} = \frac{1.195 \times 10^{-5}\ \mathrm{M}}{100} = 1.195 \times 10^{-7}\ \mathrm{M}$$

$$[\mathrm{E}]_0 = \frac{[\mathrm{E}]_{\mathrm{diluted}} V_{\mathrm{E\ added}}}{V_{\mathrm{total}}} = \frac{(1.195 \times 10^{-7}\ \mathrm{M})(0.000025\ \cancel{\mathrm{L}})}{0.0001\ \cancel{\mathrm{L}}} = 2.99 \times 10^{-8}\ \mathrm{M}$$

3.3) Putting it all together to determine turnover rate as represented by k_{obs}

Given the calculations we've made so far, we are now ready to determine the enzyme turnover rate and approximate binding affinity (Figure 5) for our mutants. We are going to put it all together to produce the observed rate constant called Kobs.

$$k_{\mathrm{obs}} = \frac{v}{[\mathrm{E}]_0} = \frac{2.30 \times 10^{-5}\ \cancel{\mathrm{M}}/\mathrm{min}}{2.99 \times 10^{-8}\ \cancel{\mathrm{M}}} = 769\ \mathrm{min}^{-1} = 12.8\ \mathrm{s}^{-1}$$

Table 5. Putting it all together: calculated average of the experimental triplicates, the initial velocity (V_0), and k_{obs}. Units for V_0 : mM min^{-1} and units for k_{obs}: min^{-1} and in sec^{-1} of product formation for our wild type triplicate set at the experimental substrate concentrations.

	Substrate concentration mM	1	2	3	Triplicate Average	Initial Velocity	*k*obs (min-1)	*k*obs (sec-1)
A	**75**	1.38E-01	1.40E-01	1.38E-01	1.39E-01	2.27E-02	7.64E+02	1.27E+01
B	**18.75**	1.12E-01	1.08E-01	1.10E-01	1.10E-01	1.80E-02	6.06E+02	1.01E+01
C	**4.69**	7.36E-02	7.08E-02	7.04E-02	7.16E-02	1.17E-02	3.94E+02	6.57E+00
D	**1.17**	2.52E-02	2.30E-02	2.50E-02	2.44E-02	4.00E-03	1.34E+02	2.24E+00
E	**0.29**	6.69E-03	5.85E-03	6.49E-03	6.34E-03	1.04E-03	3.49E+01	5.82E-01
F	**0.07**	1.89E-03	1.47E-03	1.57E-03	1.64E-03	2.70E-04	9.05E+00	1.51E-01
G	**0.02**	6.99E-04	5.41E-04	5.22E-04	5.87E-04	9.63E-05	3.23E+00	5.39E-02
H	**0**	0	0	2.30E-05	7.67E-06	1.26E-06	4.22E-02	7.04E-04

Then we can look at the relationship between the initial velocities and substrate concentrations for the data points graphically. This data relationship is the foundation to interpreting the kinetic properties of the enzyme.

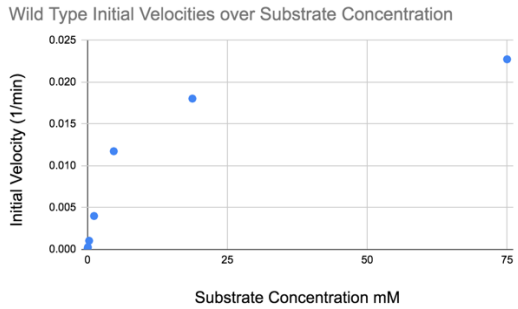


Figure 6. Scatter plot showing relationship between substrate concentration (mM) and initial velocities constant (M cm min^{-1}).

From here we will fit the data with a curve. And from this curve, we can calculate constant values that give us the ability to compare "apples to apples" and analyze the catalytic efficiency of different enzymes.

4. Modeling Kinetic Constants

The Michaelis-Menten equation (or model) describes enzymatic reactions as a function of the rate of product against substrate concentration, essentially fitting a curve to the data we produced in Figure 6. From this model we can quantify *turnover rate* and approximate *binding affinity*, which allows us to evaluate overall catalytic efficiency of our enzyme.

We like to think about enzymes reactions breaking down into two parts:

$$Enzyme + Substrate \longrightarrow Enzyme/Substrate\ Complex \longrightarrow Enzyme + Product$$

As discussed in the *Rate of Product Formation in Relationship to Substrate Concentration* section, the speed of first part (Enzyme + Substrate ⟶ Enzyme/Substrate Complex) hinges on how readily the substrate enters the active site how strongly it makes the initial connections that create the enzyme-substrate complex. The second part (Enzyme/Substrate Complex ⟶ Enzyme + Product) can be thought of as how rapidly the product is "booted" out of the active site pocket, which we call the turnover rate. We can now look at these phenomena in the context of the graph we created in Figure 7.

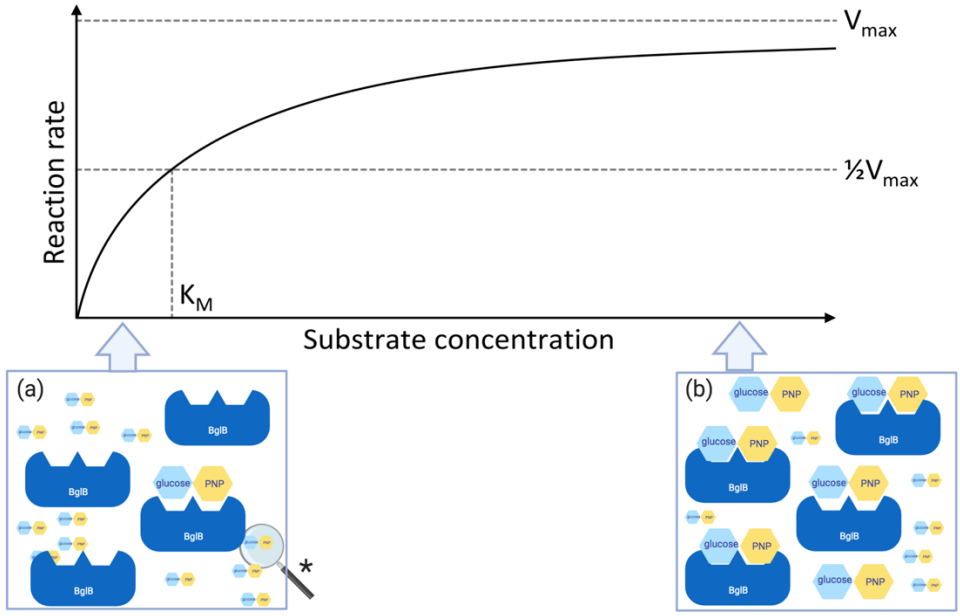


Figure 7. Linking the Michaelis-Menten model with the dogma of enzymatic reactions, where (1) substrate concentration is relatively low (a) captures the *Enzyme/Substrate Complex* formation and approximated the substrate binding affinity of the enzyme (K_M); and (2) substrate concentration is high (b) captures the *Product* formation and the turnover rate of the enzyme (K_{cat}).

4.1) Connecting Michaelis-Menten Constants to observed rate constant (k_{obs})

Then we can graph the observed rate constant (k_{obs}), against the substrate data we just created with a simple scatter plot; this is how the data is represented in the D2D app.

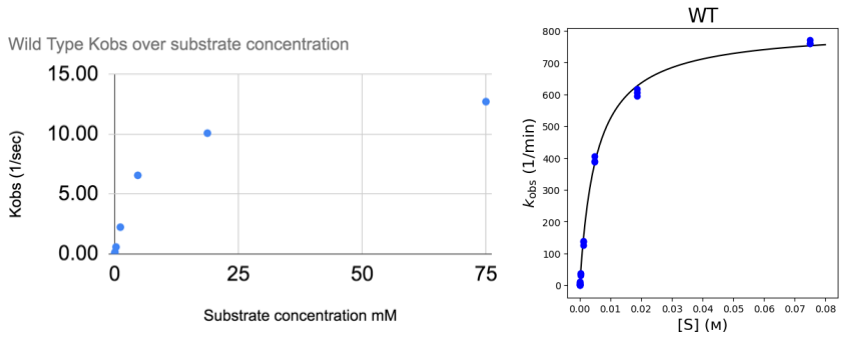


Figure 8. Scatter plot showing relationship between substrate concentration (mM) and observed rate constant (M cm sec^{-1}). In this example, k_{cat} = 807.57692 ± 75.14299 min^{-1} K_M = 5.4016 ± 0.8302 mm k_{cat}/K_M = 149.5056 ± 26.8618 $mm^{-1}min^{-1}$.

The maximum rate or asymptote produced in the k_{obs} plot is equal to k_{cat}, which is the turnover rate of the enzyme.

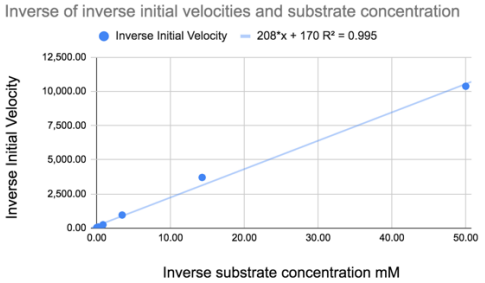
In combination, these values (k_{cat}/K_M) gives us holistic representation of the overall catalytic efficiency of the enzymes. k_{cat}/K_M is also referred to as the enzyme's **specificity constant**. The specificity constant is an indicator of how efficient the enzyme is; enzymes with high k_{cat}/K_M are efficient at what they do; They have a good balance of binding substrates and turning them over quickly.

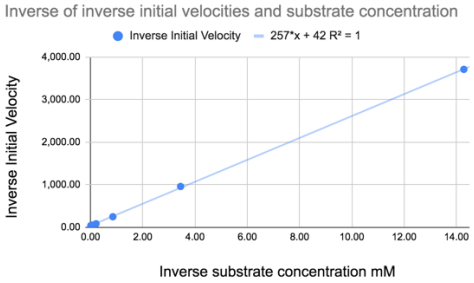
4.2) Lineweaver-Burk, linear inverse model

Unfortunately, without easy access to a non-linear regression model to fit the curve generated by the data it's not possible to proceed from Figure 8.

We will take a detour to introduce the Lineweaver—Burk model that is presented in some undergraduate biochemistry textbooks (Nelson et al., 2017) but now has only limited use in the professional field of enzyme kinetics (Dowd et al., 1965).

To use this model, we transform the data by taking the inverse of both the velocity and the substrate concentration and omitting the zero-substrate concentration (Figure 9)





$[S]^{-1}$ (M)	Velocity^{-1} (M cm min^{-1})
0.01	43.97
0.05	55.43
0.21	85.16
0.85	249.90
3.45	961.25
14.29	3,710.46
50.00	10,381.70

Figure 9. Lineweaver—Burk model used to linearize substrate and velocity data to approximate the binding affinity (K_M) and turnover rate values (V_{max}), left side includes all data points, the right side omits the data point generated from lowest substrate concentration *(italicized* in the adjacent table), which improves linear fit of the model.

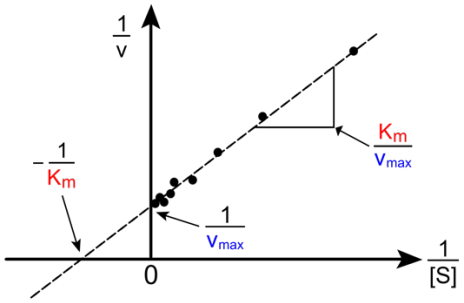


Figure 10. Graphical summary for analyzing data plotted by Lineweaver—Burk model
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4.3) Specific Activity

When you get a bottle of enzyme from the store, you will get a bottle with specific activity written on the side of the bottle and in u/mL and it's another way to calculate K_{obs}.

Extra resources

Khan Academy: Basics of enzyme kinetics graphs
Libre Texts: Michaelis-Menten Kinetics
A really great PDF guide on Michaelis-Menten Kinetics from Professor Christine Hrycyna at Purdue

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