

Lecture # 3, 4 – Selecting a Catalyst (Non-Kinetic Parameters), Review of Enzyme Kinetics, Selectivity, pH and Temperature Effects

1. Selecting an enzyme catalyst

Now that we've discussed the various enzyme classes and talked a bit about specificity, the question becomes how do we determine if an enzyme is appropriate to use as our catalyst? For a given type of reaction, it's not enough to know that we need a *lipase* or a *protease* from the hydrolase family, we need to identify one or two enzymes to carry forward for development. And even if we can narrow down the exact EC number, enzymes with the same function but from different sources will have different reaction characteristics against a particular substrate.

For design purposes, we will need to know the kinetic parameters to size a reactor and set the process cycle time. But for the initial selection process, we can choose an enzyme based on non-kinetic characteristics. By looking at several candidate enzymes, we can *screen* to determine the best choice of enzyme to use.

To set up our screen, we can put together a set of reactions that are identical except for the choice of catalyst. (Note that to be fair, the concentration of catalyst in each reaction should be the same.) We then fix the reaction time and at the end of this short batch cycle, we can take a sample and measure the concentrations of substrate and products. From these measurements, we can determine the following:

$$(1.1) \quad \text{Conversion} = X = \frac{S_0 - S_f}{S_0} \times 100\% = \frac{\sum P_{i(usur,S)}}{S_0} \times 100\%$$

$$(1.2) \quad \text{Yield}_p = \frac{P_i}{S_0} \times 100\% \quad \text{for each product, and if the products are chiral,}$$

$$(1.3) \quad \text{EE}(R) = \frac{R - S}{R + S} \times 100\%$$

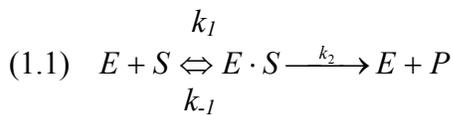
We can look at the *conversion* to get a sense of the kinetics, since enzymes that “work” faster will have converted more substrate in this fixed unit of time. We look at the *EE* to get a sense of the *enantioselectivity/specificity*. Given these two parameters, how do you choose an enzyme to take forward? While the kinetics are important for an efficient process, these can often be optimized by adjusting the reaction conditions (*eg*, pH, temperature, co-solvents, etc.). Thus, we can use our basic engineering knowledge for optimization to get the fastest reaction. What is much more difficult to control is the

selectivity. This is more likely to require a more complicated *biological* optimization rather than our standard physico-chemical based *engineering* optimization. So, all things being equal, we would like an enzyme to deliver the highest EE material possible, while also achieving “good” conversions in the initial phase. The goal of process development is to then make that good conversion better.

2. Michaelis-Menten kinetics

As with standard chemical reactions, biocatalytic reactions also have characteristic kinetics and reaction rates based on the reaction equation. As a review, consider the most commonly followed reaction kinetics, Michaelis-Menten kinetics.

Start with the following equation for the reaction:



This equation assumes that the binding of substrate and enzyme is a reversible step, and that product release from the enzyme is essentially instantaneous (not kinetically limited). Stoichiometric rate equations can be written for the substrate, enzyme, enzyme-substrate complex and product species as follows:

$$(1.2) \quad v = \frac{d[P]}{dt} = k_2[E \cdot S]$$

$$(1.3) \quad -\frac{d[S]}{dt} = k_1[E][S] - k_{-1}[E \cdot S]$$

$$(1.4) \quad \frac{d[E]}{dt} = -k_1[E][S] + k_{-1}[E \cdot S] + k_2[E][P]$$

$$(1.5) \quad \frac{d[E \cdot S]}{dt} = k_1[E][S] - k_{-1}[E \cdot S] - k_2[E \cdot S]$$

Since the amount of enzyme present is small relative to the substrate concentration (that’s why it’s a catalyst!), we can assume that Equation (5) is ~ 0 . This is the quasi-steady state approximate. Then rearrange this equation:

$$(1.6) \quad k_1[E][S] = (k_{-1} + k_2)[E \cdot S] \Rightarrow [E \cdot S] = \frac{k_1}{(k_{-1} + k_2)}[E][S]$$

Now look at the mass balance for total (observable) enzyme –

$$(1.7) \quad [E_0] = [E] + [E \cdot S] \quad \text{Note that we know the initial charge of enzyme, } E_0$$

Plugging back into Equation (6), we get the following expression:

$$(1.8) \quad [E \cdot S] = \frac{k_1}{(k_{-1} + k_2)} ([E_0] - [E \cdot S])[S] = \frac{k_1}{(k_{-1} + k_2)} [E_0][S] - \frac{k_1}{(k_{-1} + k_2)} [S][E \cdot S]$$

$$(1.9) \quad [E \cdot S] = \frac{\frac{k_1}{(k_{-1} + k_2)} [E_0][S]}{1 + \frac{k_1}{(k_{-1} + k_2)} [S]} = \frac{[E_0][S]}{\frac{(k_{-1} + k_2)}{k_1} + [S]}$$

Finally, plug back into Equation (2) to obtain the following equation:

$$(1.10) \quad v = \frac{k_2 [E_0][S]}{\frac{(k_{-1} + k_2)}{k_1} + [S]} = \frac{k_{cat} [E_0][S]}{K_M + [S]} = \frac{V_{max} [S]}{K_M + [S]}$$

This is the classic Michaelis-Menten rate equation, where the first order rate constant, k_{cat} is k_2 , V_{max} is the product of this value and the enzyme concentration, and K_M , the Michaelis constant is a mixture of the rate constants that describes the formation and dissociation of the enzyme-substrate complex. This gives a sense of the affinity of the enzyme for the substrate. Note that when $[S] \gg K_m$ (small K_m = high affinity), the reaction kinetics are first-order with respect to the enzyme concentration, and equal to the max rate V_{max} . When $[S] \ll K_m$ (large K_m = low affinity), the kinetics are first order with respect to $[S]$, and the reaction rate is k_{cat}/K_m .

There are other approximations for enzyme kinetics that lead to different rate equations, but ones that are still of the form as given in Equation (1.10), *ie*, saturation kinetics. The two parameters, k_{cat} and K_m , are the primary indicators of how well an enzyme will react with a particular substrate. In considering biocatalysis, we'd like to have enzymes that function with high k_{cat} and low K_m , *ie*, the reactions are fast with high affinity for the substrate.

To determine the parameters of interest, one can graphically represent the data. Rearrange Equation (1.10) as follows:

$$(1.11) \quad \frac{1}{v} = \frac{K_M}{V_{max}} \cdot \frac{1}{S} + \frac{1}{V_{max}}$$

A plot of $1/v$ versus $1/S$ will give a Lineweaver-Burker plot, where the slope is K_m/V_{max} and the y-intercept is $1/V_{max}$. Note that the reaction rates should be *initial* rates, determined when the substrate is in excess and essentially constant.

Lineweaver-Burke plots are considered most accurate for determination of V_{\max} since this is the value of $1/v$ as $1/[S]$ approaches zero, or for large substrate concentrations. In this case, the data tend to cluster towards the origin where the intercept is determined. The slope, from which K_M is calculated, is heavily influenced by the values of reaction rate at low substrate concentrations, *ie*, when $1/[S]$ is larger *and* when measurements of $[S]$ are likely to be less accurate. To avoid weighting the data at low substrate concentrations too much, a second type of plot can be used to calculate the parameters. By multiplying both sides of Eq (1.11) by V_{\max} and v , we can get the following expression:

$$(1.12) \quad v_{\max} = K_M \frac{v}{S} + v \Rightarrow v = v_{\max} - K_M \frac{v}{S}$$

Here, you would plot v vs. v/S to get an *Eadie-Hofstee* plot. The slope is $-K_M$ and the y-intercept is V_{\max} . This is considered a more accurate way to obtain the value for K_M since the data are more widespread.

In general, you will not know the kinetic parameters of an enzyme of interest for your (unnatural) substrate. But, if you conduct a few experiments to collect rate data, you can determine these parameters on your own.

3. Selectivity

We've already talked about one way to quantify selectivity, by measuring the EE. One can also talk about selectivity from a kinetic perspective, in determining the relative activity against desired vs undesired substrate, or for the formation of desired vs undesired product (*eg*, enantiomers) from a single substrate. Recall from 10.37, that one can define the *selectivity* of a reaction based on the kinetic parameters. So if D is my desired product and U is my undesired product, the selectivity for desired product can be expressed as the ration of the reaction rates for production of D over U:

$$(2.1) \quad E = \frac{r_D}{r_U} = \frac{(V_{\max,D} S_D) / (K_{M,D} + S_D)}{(V_{\max,U} S_U) / (K_{M,U} + S_U)} = \frac{(V_{\max,D} / K_{M,D}) S_D}{(V_{\max,U} / K_{M,U}) S_U}$$

if we assume that the K_M values are significantly higher than the substrate concentrations, or if we simply make a first-order approximation for the reaction kinetics. In both cases, there is a single enzyme, so the equation can be re-written as follows:

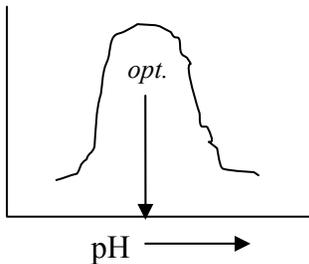
$$(2.2) \quad E = \frac{(k_{cat,D} / K_{M,D}) S_D}{(k_{cat,U} / K_{M,U}) S_U} \approx \frac{(k_{cat} / K_M)_D}{(k_{cat} / K_M)_U}$$

If the initial substrate is the same and we are looking at selectivity for production of an enantiomer, then the S terms cancel, and the selectivity is simply the ratio of the first-order rate constants, k_{cat}/K_M for each product. (Note that since K_M incorporates k_2 , these terms do not cancel each other out.) If the starting substrate is a racemic mixture, then the initial concentrations of each substrate are equal and the S terms again cancel one another out. This is only true at the beginning of the reaction.

4. pH and temperature effects on enzyme activity, enzyme stability

We previously discussed the importance of composition of the polypeptide backbone, *ie*, the chemical nature of the side chains, in discussing specificity. This is also important in understanding the impact of pH and temperature changes on enzyme activity and enzyme stability.

Recall that enzymes are generally unstable when exposed to extremes of pH. The effects of pH are most easily understood in looking at the number of ionizable side groups among the amino acids. (Note that the actual pK of each group is influenced by the neighboring residues, so the numbers in the handout should be considered as a guide only.) Given that ionic interactions are one of the forces that help to establish the active conformation of an enzyme, it's clear that extremes in pH will affect the ionization state of the side groups and thereby lead to disruptions of those forces. As a result, all enzymes have some optimal pH at which they operate. A plot of activity vs pH will look something like this:



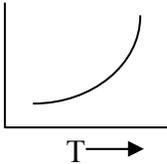
The actual “optimum” may be a range whose width varies by enzyme, but in general, these trends will hold true. Looking again at the serine proteases, three residues are important in forming the “catalytic triad” for these enzymes: Asp, His, and Ser.

The Serine residue attacks the peptide bond to form an acyl-enzyme intermediate. Bond cleavage is then assisted by donation of a proton from the neighboring Histidine residue, which is also thought to increase the ionic character of the Ser. The Asp residue also stabilizes the positive charge of the His. It is clear in this case that extremes of pH will affect the protonated state of all three ionizable groups.

The effect of *temperature* on enzyme activity is less straightforward. Enzyme-catalyzed reactions respond to changes in temperature in much the same way that non-biocatalyzed reactions respond, *ie*, with an Arrhenius relationship:

$$k = Ae^{-E_{act}/RT}$$

Remember that an enzyme serves to increase the reaction rate of the enzyme, and that the reactants (substrates) must achieve a certain energetic state to become activated. Enzymes reduce that barrier to activation; however, increasing the temperature (*ie*, the internal energy) means that more substrate is likely to have achieved the energetic state necessary for the reaction to proceed. Thus, a plot of activity vs temperature for an enzyme-catalyzed reaction looks much like one for any other reaction:



However, one is limited in the ability to use high temperatures in order to increase the activity of enzymes because of a process known as *thermal deactivation*. This process may be reversible, with activity returning with a return to optimal operating conditions, or irreversible. Typically, reversible deactivation is the result of partial denaturation of the protein from its active folded conformation. This step is also sensitive to changes in pH. Irreversible deactivation often happens as a result of protein aggregation or precipitation.

Given that there is both an enhancement of reaction rate with increasing temperature *and* a loss of enzyme due to deactivation, these two conditions must be balanced. If one were to look at the effect of temperature on enzyme activity as a function of, say, total conversion over a fixed unit of time, instead of as an initial reaction rate, the profile would not be of the form of the Arrhenius equation, but instead, would look much like the plot for pH optimum presented above.

Note that the temperature optimum of an enzyme is usually quite similar to the environmental temperature at which they enzyme is found in nature. Thus, enzymes from mammalian sources tend to have T_{opt} 's around 37 °C, *ie*, body temperature. This fact can be exploited by prospecting for enzymes amongst organisms that live at extremes of temperature, so-called *thermophiles*.

It should be remembered that all enzymes have a certain half-life, even when operating at the optimal temperature. It is therefore important that a biocatalyst have suitable properties to ensure activity over the time needed to complete the conversion. We'll come back to this when talking about the design/development process.