Lecture # 9, 10, 11 – Engineering Solutions to Biocatalysis Design Problems

Handouts: (1) Bornscheuer paper [TIBTECH.2002 20(10):433], (2) Guide Sheet, (3) Immobilized Enzyme Kinetics [Bailey and Ollis, pp. 202-217], (4) Yield Improvement Examples

Now that we've talked about finding purified enzymes or whole cells to perform a bioconversion, how that selection is made, and how you would optimize your conversion, we need to address possible issues that may arise and how we would find solutions to them. We will cover this topic over several lectures, and will think about this in a couple of ways. First, we can encounter issues of a *biological* nature, meaning those issues that are an inherent part of the enzyme and its behavior. We may also encounter problems of a *physico-chemical* or what we'll consider *engineering* nature, meaning those problems that are not inherent to any particular enzyme but reflect characteristics of the substrates, products, or inherent properties (energy) of the chemical reaction. For some of these design challenges, we will be able to apply *engineering solutions*, meaning the catalyst will be fixed and we will adjust our design to deal with the problem. For others, we will need to apply *biological solutions*, meaning the catalyst itself will need to be altered. For this set of lectures, we'll focus on those problems that have engineering solutions. We will then review our process design/development approach for purified enzymes and whole cell catalysts, and finally, to end the course, come back to a discussion of biological solutions for process design challenges.
You can use the "Guide Sheet" handout to keep track of the design issues we'll discuss and the solutions we're proposing to solve them.

1. General Design Issues

A. Acidic or basic product formation (Engineering problem)

This is really a very straightforward concept. The key issue here is to know whether you need a buffered process or a pH-controlled process. If your substrates and products are not sufficiently acidic or basic, a buffered process will suffice. In this case, you will establish a reaction pH in a buffered solution, and you can operate the reaction phase as a closed process, with no addition of acid or base throughout the cycle. If, however, you have a product or substrate that is (usu.) acidic, then you will probably need a pH-controlled reactor. Consider the hydrolysis of esters to acids. Depending on whether the hydrolyzed product is a strong acid, buffering may not be sufficient and you will need to add base to keep the reaction pH in the previously identified operating range. From a development perspective, you can measure the pH as a function of reaction time to determine whether or not the pH is changing to a level where enzyme activity will be

affected. From a design perspective, you then need to make sure you have the equipment for a pH control and that you supply enough base to sustain the entire reaction.

B. Exothermic reactions (Engineering problem)

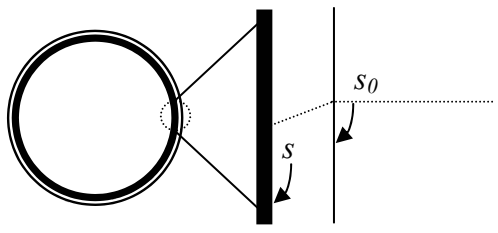
The principle of exothermic (or endothermic) reactions is the same as for pH – you will need to exercise temperature control to maintain the best reaction environment. The one aspect of a bionconversion in which you will always need to have temperature control is for the growth phase of a whole cell conversion. Cell growth is a very exothermic reaction, and without proper cooling of the vessel, temperatures can spike very rapidly, certainly affecting gene expression (particularly of "heat shock" proteins) and possibly causing cell death. Generally speaking, all reactions, unless they are run at room temperature, will be conducted in a temperature-controlled vessel. Again, what you want to remember here is to just be aware of the phenomenon.

C. Enzyme instability (Biological problem)

Now, we can talk about a challenge whose solution may require that we alter the assumptions that enter into our design process. As mentioned in our discussion of enzyme stability (Lecture 3), all enzymes have some inherent instability, usually described by the half-life, *ie*, the amount of time after which half of the enzyme's activity has been lost. Some enzymes, however, are so unstable, that their use is not very practical for industrial purposes. One engineering solution to the problem of instability is *immobilization*. In many cases, fixing the enzyme to a solid support improves the stability and increases the half-life of the preparation relative to the enzyme in free solution. There are several theories for how this stabilizing effect occurs, which we won't cover but that can be found in several textbooks. Immobilization also allows the enzyme to be retained in the reactor, for example, if you had a plug flow configuration and wanted to operate continuously.

If your enzyme is immobilized, you must now combine considerations of *mass transport* to the enzyme-containing particle in addition to the reaction (*ie,* 10.302 meets 10.37). At the extremes, the reaction will be either reaction-limited or transport-limited. If the reaction is transport-limited (or significantly influenced by the transport properties), then your kinetics will not be of the Michaelis-Menten form, but will be best represented by the substrate transport rate.

Consider first the case of an enzyme immobilized to the surface of a non-porous solid support. In this case, we can assume that a thin film (boundary layer) exists around the particle, with bulk substrate concentration, s_0, at the edge of the boundary layer; concentration, s, at the surface of the particle; and a linear gradient across the thin film layer. (Assuming non-charged support.)



At steady-state, the flux to the surface of the catalyst particle is equal to the reaction rate. If we assume Michaelis-Menten kinetics, we can then write the following equation:

$$(1.1) \quad N_s = k_s\left(s_0 - s\right) = \frac{\bar{v}_{\max} s}{K_m + s} = \bar{v}$$

where v(bar) is a surface reaction rate, *ie*, rate per unit surface area. You can introduce dimensionless variables to reduce the total number of parameters from four (V_{max}, K_m, k_s, and s_0) to two:

$$(1.2 - 1.4) \quad x = \frac{s}{s_0}, \kappa = \frac{K_m}{s_0}, Da = \frac{\bar{v}_{\max}}{k_s s_0} = \frac{maximum \cdot rxn \cdot rate}{maximum \cdot transport \cdot rate}$$

Da is the Damköhler number. If Da << 1, then the reaction is reaction-limited, and if Da>>1, the reaction is transport-limited. If we use the dimensionless variables in Eq (1.1), we get the following expression:

$$(1.5) \quad \frac{1-x}{Da} = \frac{x}{\kappa + x}$$

This equation is quadratic in x and can be solved analytically; however, it contains parameters that cannot be observed directly in a system that has both reaction- and transport-influenced kinetics. Instead, engineers use an *external effectiveness factor* to describe reaction kinetics with external mass transfer effects. The external effectiveness factor is obtained by looking at the observed reaction rate in the absence of mass transfer effects, when $s=s_0$ and $x=1$. Under these conditions, the observed reaction rate can be expressed as the right-hand-side of Eq 1.1 as follows:

$$(1.6) \quad \bar{v} = \frac{\bar{v}_{\max} x}{\kappa + x} = \frac{\bar{v}_{\max}}{1 + \kappa}$$

The effectiveness factor can then be defined as follows:

$$(1.7) \quad \bar{v} = \eta_E \left(\frac{\bar{v}_{\max}}{1 + \kappa} \right),$$

where $0 \le \eta_E \le 1$, and is a ratio of the observed reaction rate to the rate in the absence of mass transfer effects. This can also be defined as follows (from Eq 1.5 and x=1):

$$(1.8) \quad \eta_E = \frac{observed \cdot rxn \cdot rate}{rate \cdot w/out \cdot mass \cdot transfer \cdot resistance} = \frac{\frac{x}{\kappa + x}}{\frac{1}{\kappa + 1}} = \frac{x(\kappa + 1)}{(\kappa + x)} = \frac{(1-x)(1+\kappa)}{Da}$$

Now look at the extremes:

1. If Da >> 1, then the reaction is transport limited, and x is small. So Eq (1.8) becomes:

(1.9) $\eta_E = \dfrac{1+\kappa}{Da}$, and Eq (1.7) becomes:

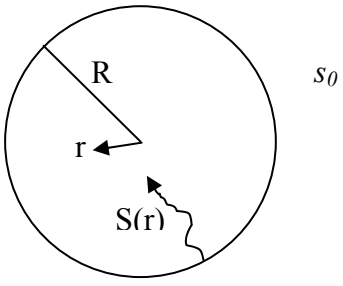
(1.10) $$\bar{v} = \left(\frac{1+\kappa}{Da}\right)\left(\frac{\bar{v}_{\max}}{1+\kappa}\right) = \frac{\bar{v}_{\max}}{\bar{v}_{\max}/k_s s_0} = k_s s_0$$

This says that the reaction is transport limited and is first-order with respect to the bulk substrate constant, but the reaction rate is actually a *transport* rate. This system would not obey Michaelis-Menten kinetics.

2. If Da<<1, then the system is reaction rate limited, and x is roughly equal to 1. In this case Eq (1.8) says that $\eta_E = 1$ and the system behaves as if there are no mass transfer limitations.

In practice, there are plots of η_E as a function of κ and Da that allow you to estimate the various parameters in such systems.

We are actually much more likely to use a porous catalyst for an immobilized enzyme system than a non-porous one. *Why? Because the surface area to volume ratio is greatly increased*. In this case, we will assume that there are no external mass transfer limitations, such that the concentration at the surface of the particle is equal to the bulk substrate concentration, s0, and that the diffusion rate of substrate through the porous catalyst can be represented as a function of an effective diffusivity, De. (Again, assume non-charged support.)



The steady-state mass balance (across a spherical catalyst shell) in this case becomes:

(1.11) $$J(r) = \frac{D_e}{r^2}\left[\frac{d}{dr}\left(r^2\,\frac{ds(r)}{dr}\right)\right] = \frac{v_{\max} s(r)}{s(r)+K_M} = v(r),$$

where V_{max} is the reaction rate per vol particle.

This becomes:

$$(1.12)\quad D_e\left(\frac{d^2s}{dr^2}+\frac{2}{r}\frac{ds}{dr}\right)=\frac{v_{\max}s(r)}{s(r)+K_M}$$

We'll again use dimensionless variables to represent this more generally, with s(bar)=s/s_0, r(bar)=r/R, and $\beta=s_0/K_M$, to get the following:

$$(1.13)\quad \frac{d^2\bar{s}}{d\bar{r}^2}+\frac{2}{\bar{r}}\frac{d\bar{s}}{d\bar{r}}=\frac{vR^2}{D_e s_0}=9\phi^2\frac{\bar{s}}{1+\beta\bar{s}}$$

$$(1.14)\quad \phi=\frac{R}{3}\sqrt{\frac{v_{\max}/K_M}{D_e}}$$

where ϕ is the Thiele modulus. This is analogous to the Thiele modulus defined for heterogenous catalysis in 10.37. The square of the modulus is similar to the Damköhler number in that it represents a reaction rate divided by a diffusion rate. If ϕ is large, the system is diffusion limited, and if it's small, the system is reaction-limited. The boundary conditions for this differential equation are that s(bar)=1 at r(bar)=1, and the derivative is 0 at r(bar)=0.

We can also once again define an *internal effectiveness factor,* η_I, as the observed reaction rate divided by the rate in the absence of internal concentration gradients, or when the internal substrate concentration is equal to the bulk substrate concentration. Just as with non-porous catalysts, the effectiveness factor can be defined as follows:

$$(1.15)\quad v=\eta_I\left(\frac{v_{\max}\beta}{1+\beta}\right)$$

From this, we see that $\eta_I = f(\phi,\beta)$. And as usual, we would need to solve this numerical or refer to plots that express the internal effectiveness factor as a function of the Thiele modulus and beta. We could also write an expression similar to Eq 1.8 if we set the observed reaction rate equal to the substrate flux into the pellet. We instead define an *observable* Thiele modulus in terms of parameters that are measurable as follows:

$$(1.16)\quad \Phi=\frac{v_0}{D_e s_0}\left(\frac{V_p}{A_p}\right)^2$$

Here V_p is the particle volume and A_p is the surface area. In practice, then, you can refer to a plot of the effectiveness factor as a function of the observable modulus for various values of β to estimate numerical values for the effective diffusivity based on observed reaction rates as a function of particle size (see Figure 4.21 as an example). Note that the

Thiele modulus (effective and actual) is a function of particle size, and for small R, the system is reaction-limited, meaning you will cease to see differences in reaction rate as a function of particle size.

2. Substrate/Product Specific Issues

A. Substrate solubility (Engineering problem)

Problem – Substrates that are sparingly soluble in water present a two-fold problem for bioconversions. First, the low aqueous-phase concentration means that the reaction rate will be limited by the solubility and you will not likely be able to reach the maximum reaction velocity, V_{max}. Second, the low solubility means that your *per batch* yield may be limited by the amount of substrate that can be dissolved.

Solutions – Low substrate solubility can be addressed in several ways:

- **Two-phase system** – Compounds that have low aqueous phase solubility usually have good organic solubility. Therefore, you can operate the reactor as a two-phase system in which the substrate is added in an organic phase (for a liquid-liquid system) and the enzyme is present in the aqueous phase. The concentration of substrate in the aqueous phase will be less than or equal to the solubility limit. One measure of the solubility in water is the octanol-water partition coefficient:

  (2.1) $$K_{ow} = \frac{C_o}{C_w}$$

  Octanol is considered a good proxy for many water-immiscible organic solvents. The concentration in water is not equal to the solubility in water, since the water will pick up some of the organic phase and vice versa. If the substrate is itself an organic liquid (like the indene example), it can form the second phase, and the aqueous phase concentration will be the solubility limit (or saturation).

  The second phase can also be a *solid* phase. An example of a solid phase substance that readily adsorbs organic compounds is charcoal, also known as activated carbon. One of the final report assignments uses a solid phase to deliver substrate to the enzyme.

  Note that with a two-phase system, particularly a liquid-liquid one, you must be cautious that the enzyme is not severely destabilized in the presence of an organic phase. Most lipase enzymes require a two-phase system for proper functioning, but many others will denature at the organic-aqueous interface.

- **Substrate feeding/fed-batch reactor design** – If a two-phase system does cause denaturation of the enzyme or results in a substrate concentration so high as to be toxic, the substrate can be added to the reactor over time instead of in a single dose. This is a *fed-batch* reactor configuration. By using a fed-batch reactor, the total amount of product obtained per batch can be increased; however, note that

this is no longer a constant-volume system. As substrate is added to the system, the volume will increase as a function of time as follows:

(2.2) $V(t) = V_i + F_0 t$

where V_i is the initial volume and F_0 is the flow rate into the reactor. The drawback to a fed-batch system is that the substrate concentration in the reactor decreases as the flow-rate increases for a constant feed concentration since the volume is increasing. From a practical standpoint as well, a fed-batch reactor requires that the initial working volume be lower than a batch reactor to be able to accommodate the additional liquid added to the system.

Note: Fed-batch reactors are usually only operated with liquid feed streams. Powders are considered an occupational hazard, esp. in large amounts.

- **Ionic liquids** – Ionic liquids are non-flammable, non-volatile organic salts. They are like organic solvents, but are charged and so also have hydrophilic character. This should allow for increased solubility of polar substrates with retaining some enzyme functionality. Research into the use of ionic liquids for enzyme reactions is fairly young, but it provides a potential solution for enzyme reactions with sparingly soluble substrates. Note that the reference article on optimizing lipase reactions discusses the use of ionic liquids.

Note that product solubility (or insolubility) is not a problem. Why not??? Because it aids in the isolation process!

B. Substrate inhibition/toxicity (Biological problem)

Problem – From our previous study of inhibition kinetics, we saw that substrate inhibition is a unique case of uncompetitive inhibition in which increasing the substrate concentration decreases the reaction rate. In this case, the rate expression for Michaelis-Menten kinetics is as follows:

(2.3) $$v = \frac{v_{\max}[S]}{K_M + [S]\left(1 + \frac{[S]}{K_{S2}}\right)} = \frac{v_{\max}[S]}{K_M + [S] + {[S]^2}/{K_{S2}}}$$

Solution – This rate expression says that the maximum reaction rate is lowered, and that the highest rate would occur at a relatively low substrate concentration. From 10.37, you learned that which reactor configuration would give the lowest average reactant concentration? Answer: CSTR. A traditional CSTR is one in which the outlet composition is representative of the reactor composition. This is not a good idea for a bioconversion with free enzyme. Describing the reason for this, *and thinking of some other possibilities*, is left as a homework exercise. Suffice it to say that we have already

talked about solutions to other challenges that would also be appropriate for addressing the challenge of substrate inhibition.

C. Product inhibition/toxicity (Biological problem)

Problem – Product inhibition results in an upper limit on the amount of achievable product concentration in the reactor as product accumulation leads to a drop in reaction rate. It is most often recognized as a plateau in the product concentration at a level well below the desired conversion (usu >95%). Note that substrate inhibition is evident by a decrease in *initial* reaction rate as the starting substrate concentration is increased, while product inhibition can be seen in three ways: as a reduction (1) in *initial rate* in the presence of added product, (2) in the overall productivity in the presence of added product (Buckland paper), or (3) in the *reaction rate over time* as product accumulates. In the third case, you'd have to account for the reduction in rate that would occur with a drop in substrate concentration.

Solution – As with the case of substrate inhibition, the solution to product inhibition is to keep the product concentration low. Two ways to accomplish this are as follows:

- **Limit the substrate concentration to limit the product concentration** – Since product is made from substrate. Limiting the substrate concentration will limit the product concentration. This has a tremendous disadvantage – it limits the per batch productivity of the system.

- **Actively remove product as it's made** – This is called "*in situ* product removal" and it combines the isolation phase with the reaction phase. In the simplest manifestations, the product is selectively removed from the reaction mixture into a separate phase. This may be a liquid-liquid system in which, for example, the product is less polar, or a solid-liquid two-phase system where the product is selectively adsorbed. This can be simply thought of as the opposite of using a two-phase system to *deliver* substrate in the case of low aqueous-phase solubility. As in that case, the octanol-water coefficient is a useful parameter to determine the partitioning of product between aqueous and organic phases. The same design assignment that used a two-phase system to deliver substrate also used that same system to remove product!

3. Yield Improvement Issues

A. By-product formation (Biological problem)

Problem – The main drawback to by-product formation is yield loss. In most cases, substrate that is converted to an undesired by-product cannot be recovered back to the desired product. Therefore, while increasing the catalyst loading can increase conversion, it cannot change the yield to reduce by-product formation. The best way to deal with by-product formation is in the catalyst selection phase; however, if the only

viable catalyst also results in lots of by-product formation, there are some ways to address this problem.

Solutions – The best solution to excessive by-product formation is a biological solution. We will cover this later, but know that it is a time-consuming option, and is not always successful. Two engineering solutions are:

- **Optimization of the growth phase** – Recall that the enzymes producing both desired and undesired compounds are made in the growth phase. Therefore, it is possible that optimization of the growth phase can reduce production of the undesired products. This is not as easy as it sounds. If you don't know which enzyme is responsible for the undesired activity, it's difficult to know when and how it's produced, *ie*, constitutively, inducibly, in response to excess or starvation, etc. It is very possible that the enzyme is always produced, and that there is no reasonable modification to the growth medium that can be made that will selectively reduce production of that enzyme while maintaining good growth.

- **Selective inhibition of undesired reactions** – In this solution, we can use enzyme inhibition to our advantage. If we can selectively inhibit the enzyme producing the undesired reaction, then we can minimize yield loss to those by-products. Consider again Whole Cell Case Study #1 (Yield Improvement Handout, part 1). Based on the products profile, a reaction pathway was proposed by which the enone could go to allylic alcohol or saturated ketone, and saturated ketone was further reduced to saturated alcohol. Reducing the amount of saturated ketone produced would then reduce the levels of two undesired by-products. Therefore, the researchers searched for compounds that would selectively inhibit the production of sat'd ketone.

  Of the three types of inhibition, which would you prefer to reduce by-product formation? A competitive inhibitor is desired in this case because you want a compound that will prevent binding of the substrate. Since competitive inhibitors occupy the enzyme's active site, they are most likely to be structurally similar to the substrate. In this case study, an α,β-unsaturated carboxylic acid structure instead of ketone was scanned, and cinnamic acid was identified as an inhibitor that reduced the production of undesired by-products. The yield improved from ~45% to ~75% in this way. Note that selective inhibition requires a good understanding of (or at least a good hypothesis for) the reaction pathways to guide the selection of an inhibitor. Otherwise, there're far too many chemicals out there! Additionally, as a practical matter, this method requires that the most effective inhibitor also be cheap enough for a economically viable process.

B. Equilibrium reactions (Engineering problem)

Problem – Equilibrium reactions are defined in this case as those with an equilibrium constant near 1. This is a particular drawback of the Class 2 Transaminase enzymes. If K_{eq}~1, then the yield will be near 50%, not very good for a bioconversion.

Solution – The solution to an equilibrium bioconversion is the same as for non-bio reactions: you need to remove the product to drive the reaction.

- **In situ product removal** – We've already discussed this in talking about a solution to product inhibition. The same principles apply here, you can use a two-phase system (L-L or S-L) to selectively remove product from the aqueous phase.

- **Coupled reactions** – Consider the case of the transaminases (Yield Improvement handout, part 2). Transaminases convert keto acids to amino acids, using another amino acid as the amine donor. Glutamate is a common amine donor for this class of enzymes. In this example, the α-ketoglutarate co-product is converted back to glutamate using a second transaminase with aspartic acid as the amine donor. De-amination of aspartic acid produces oxaloacetate, which spontaneously decarboxylates to form pyruvate. The key to this sequence is the last step – OAA to pyruvate is a "dead-end" reaction, which drives further glutamate production to keep making OAA, which in turn drives further desired product formation to consume glutamate. In this way, the desired reaction can be driven to completion.

C. Yield limit of resolutions (Engineering problem)

Problem – Recall that resolutions are defined as the selective conversion of one enantiomer within a 50-50 (racemic) mixture. Thus, the natural yield limit of these reactions is also 50%, but for different reasons than equilibrium reactions.

Solution – Racemize either the unreacted enantiomer, *ie*, convert the undesired substrate to the desired form, or the reacted product, to provide "fresh" substrate for reaction.

- **Racemization of unreacted substrate** – This is called "dynamic kinetic resolution (DKR)" and it depends on the existence of a fast-acting racemization step. Once product is formed and the EE moves from ~0%, the racemization reaction results in an equal mixture again. This continuously supplies new substrate, driving the theoretical yield towards 100%.

- **Racemization of reacted product** – Here, you've selectively removed the undesired enantiomer through enzymatic conversion, and now you want to recycle the product to re-form the substrate so that it can be reacted again. The recycle scheme in the handout is an example of this (Yield handout, part 3). Note that this combines DKR with additional steps (namely, esterification) for recycle.

The methodology for both of these cases is the same, and generally, speaking, the racemization steps are accomplished through *chemical* conversion rather than enzymatically. This presents another set of engineering challenges: how to find a set of reaction conditions that allow for both chemical and biological catalysis in the same vessel *or* set up a two reactor system. That's a topic for another course!