10.492 - Integrated Chemical Engineering (ICE) Topics: Biocatalysis
MIT Chemical Engineering Department
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Lecture #6 – Whole Cell Biocatalysts: Enzyme Production (The Growth Phase); By-Product Formation, Substrate/Product Inhibition and Toxicity (The Conversion Phase)

Handout: Whole Cell Conversion Case Studies

Recall that one of the things that makes a whole cell process different from a purified enzyme process is that in the former case, we now have two phases of process design: the growth phase and the conversion phase. We'll consider each of these phases in turn, but remember that in all cases, the "active" ingredient is an enzyme and the characteristics of enzymes that we've studied to date will still apply.

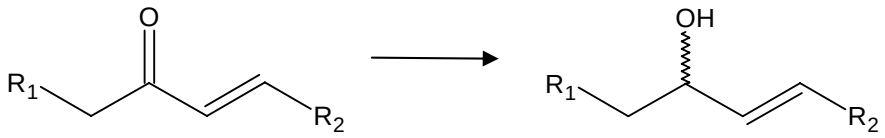
1. The Growth Phase → Enzyme Production

The growth phase is in many ways the most critical part of a whole cell bioconversion process because it's during this phase that the enzyme in which we're interested will be produced. In the "ideal" scenario, the enzyme will be produced throughout all phases of the growth cycle and its production will not depend on the presence of any other compound in the growth medium. In the most common scenario, this is far from the truth! We will not be able to go into all of the details of how one would design or optimize a growth medium to maximize production of the enzyme of interest (a large but critical task). Instead, we will focus on a few specific ways to optimize enzyme production, including: pH and temperature optimization, inducible gene expression (positive control), and overcoming catabolite repression (a form of negative regulation of gene expression).

A. Optimizing pH and Temperature

In considering pH and temperature, this is conceptually straightforward. Typically speaking, microorganisms will grow optimally at the temperature and pH of their naturally-occurring habitat. We assume that evolution is pretty efficient and that the bugs will have been optimized for home growth! A bacterium like *E. coli*, which is naturally found in the human gut, will grow at body temperature (37 °C) and roughly neutral pH (usu. 7.2-7.5). On the other hand, many yeast tend to thrive at lower temperatures (30-35 °C) and acidic pH (~4-5). While generally speaking, the physical conditions that favor optimal *growth* of the organism will also favor production of the desired enzyme, this is not always the case. Therefore, although a whole cell process requires that you optimize both growth and conversion phases, you cannot do one independently of the other Experimentally, this is accomplished by testing the growth of the organism under a variety of conditions, and then observing how well the resulting cells perform the conversion. NOTE THAT THE GROWTH CONDITIONS CAN AFFECT NOT JUST

THE CONVERSION (IE, THE SPECIFIC ACTIVITY OF THE CATALYST) BUT ALSO THE EE!!! As an example, consider this reaction from Problem Set #2, Problem #5 (Courtesy of Merck & Co., Inc. Used with permission.):



This bioconversion was accomplished using a yeast known as *Candida chilensis*. [Refer to handout on case studies.] If you look at the data obtained for the bioconversion activity of this organism as a function of growth pH, you'll find that the highest EE was obtained when cells were grown at pH=4.5, and that EE decreased as pH increased. This may seem like a bizarre result, and you may be thinking, why would the growth pH affect the specificity of an enzyme? (You may especially think this if you realize that unlike temperature, internal and external pH's are often not balanced. So even if the growth medium has a pH of 4.5, the internal environment of the cell is closer to neutral and is usually fairly insensitive to the external environment.) Good question! The answer here is that we don't know if the pH affects specificity, but most likely, it does not. You have to remember that in a whole cell system, you have *lots* of other enzymes in addition to your desired enzyme, and those other enzymes may also operate on your substrate. So, a more likely scenario than the external pH affecting the specificity of a particular enzyme is the expression of *alternate* enzymes at different pH's that will also convert the substrate to product, but not with the same desired specificity. This type of phenomenon can be observed in the second case study, where four different enzymes are believed to act upon a single substrate (red, black, yellow, and green arrows). Additionally, an "EE" upgrade phenomenon was observed here in which the undesired enantiomer was selectively removed (blue arrow pathway) by another enzyme. Therefore, if you can find a set of conditions that favors expression of the enzyme that you want over others, you can improve the performance of your system.

Beyond optimizing pH and temperature (and not considering full-scale optimization of the medium), we also need to know if there are medium components whose presence or absence is required for conversion. We are usually talking about the phenomena of *inducible gene expression* or *catabolite repression* of gene expression in these cases.

B. Inducible Gene Expression (Positive Control)

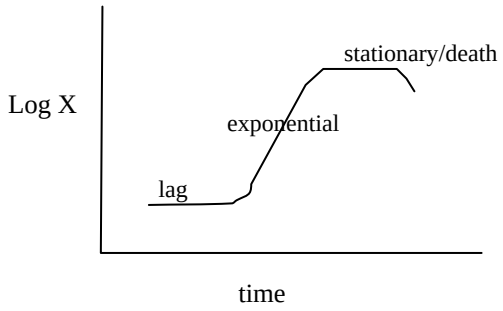
[Find out familiarity with the concept of inducible expression.] In the case of positively-controlled inducible gene expression, the cells need to be exposed to a particular compound in order for the desired enzymes to be produced. As an example, consider again the Buckland et al, *Metabolic Engineering* paper. In this case, the researchers chose to use a strain of *Pseudomonas putida* to oxidize indene because it had been documented in the literature that the strain was capable of performing this reaction. However, the conversion only happened in the presence of toluene. Therefore, the enzyme responsible was deemed a toluene-inducible dioxygenase. This also means that in order to get production of the desired bioconversion activity (*ie*, the enzyme of

interest), toluene had to be supplied to the cells to induce expression. It's hard to know whether an enzyme requires induced expression – after all, you will only see expression (activity) if the enzyme is produced, and you may not always know what the required ingredient was in the medium that resulted in expression. In this case, then, you will typically need to rely on the published literature to make a best guess as to whether production of your enzyme is induced or not. Note in this case, though, that there was a solution to toluene-induction – identifying a mutant capable of making the enzyme without toluene present.

C. Catabolite Repression (Negative Control of Gene Expression)

The case of toluene-inducible expression is an example of positive control of gene expression. Catabolite repression is an example of *negative* control of gene expression, where the enzyme is not produced while a compound is still present in the medium, but will appear after that compound is gone. Catabolite repression is typically observed with glucose. In many organisms, including bacteria and yeast, cells will selectively metabolize glucose over other carbon sources, and while glucose is present, the cell will not produce any enzymes that are needed to utilize those other sources. If it turns out that the enzyme you need is one of those that suffer from catabolite repression, then you will not observe any activity while glucose is still present in the medium. As an example, go back to our first case study, the reduction of an α,β-unsaturated ketone to an alcohol. In this case, the conversion only occurred when glucose had been reduced to non-measurable levels. Unlike positive control of gene expression from the addition of an inducer, catabolite repression is fairly easy to measure. You can simply take a sample of cells from the exponential growth period when glucose is present, and from the stationary phase, when glucose has been depleted, and compare the *specific activity* of the two samples. Remember, that you will want to normalize to the amount of biomass present so that you are comparing against the same standard.

Once you know what components are necessary for enzyme production. You need to grow the cells. Recall from 10.37 that cell growth occurs in three phases: a lag phase, an exponential phase, and a stationary/death phase.



The lag and stationary/death phases will need to be determined experimentally. During the exponential phase, growth is autocatalytic and is represented as follows:

$$(1.1)\quad \frac{dX}{dt} = \mu X,\ \mu = \frac{\mu_{mzx} S}{K_S + S}$$

The μ term is the specific growth rate and in this M-M form is the Monod expression. It behaves as if a single enzyme were limiting growth of the organism, and so it looks like M-M kinetics. In reality, we usually operate the largest fraction of the growth cycle under the exponential phase, where μ is a constant and no substrates are limiting. So Eq (1.1) can be used to calculate the cycle time for exponential growth. Adding this time to the lag and stationary phase times will give you the cycle time for enzyme production.

The final aspect of the growth phase optimization will be to determine the maximum amount of biomass that can be obtained under the conditions that favor max production of your enzyme of interest. This will give you an idea of the upper limit of enzyme availability. Combined with the reaction optimization results, you'll use this information to determine how many reactors (or how large of a single reactor) you would need to use to convert the desired amount of substrate.

2. The Conversion Phase → Product Formation

For optimization of the conversion phase, there are a few things to keep in mind. (1) The optimal growth conditions may not be the same as the optimal conversion conditions. (2) If you have excessive by-product formation, you may need to find a way to divert substrate towards your desired enzyme and away from others. (3) If you have substrate or product inhibition/toxicity, you will need to find a design solution for this problem.

A. Optimizing the Conversion Conditions

In some cases, the conversion will take place along with growth, in which case, your growth optimization is tied to conversion optimization. However, you may also have a case where growth and conversion are de-coupled and each one is optimized independently. At this stage, optimization of the conversion is much as it would be for a free enzyme. We focus first on temperature and pH. Temperature optimization is usually very simple – remembering that enzymes are generally optimized for the temperature at which the cells grow, it is usually sufficient to have the same conversion temperature as you had growth temperature. pH, however, is another matter. As an example, consider case study #1 again [slide 3]. In this example, growth and conversion were de-coupled. EE was dependent upon the pH of the growth medium during the production phase, with higher pH's leading to lower EE's. However, if you look at the data obtained with resting (*ie,* non-growing) cells, you can see that the best conversions and EE's were obtained with pH closer to neutral. In this case, cells *grown* at pH-7 gave an EE less than 20%, but cells *reacted* at pH-7 gave EE>95%. Note that in the reaction phase, the pH could affect the functioning of the enzyme, particularly if the cells have been permeabilized (possibly by the substrate). It can also affect the substrate and the manner in which it is presented to the enzyme. The key here is to remember that pH is a factor that should be explored.

B. By-Product Formation and Side Reactions

The most important thing to know about by-products in whole cell conversions is that you have to know whether they exist! By-products can have two effects: (1) [almost a guarantee] They will affect your yield. Unless the step towards by-product formation is reversible, and is driven by consumption of the substrate by your desired, unidirectional pathway, by-products represent lost matter that is unlikely to be returned. As an examples, consider case study #2 – the initial substrate is acted upon by 4 different enzymes to make 4 different products, only two of which are desired. In the initial *Rhodococcus* sp. bioconverision, EE was greater than 95%, but yield was only about 25% because of the large number of by-products. You need to do a mass balance as soon as possible when optimizing whole cell bioconversions to determine whether or not you are making excessive amounts of by-product. (2) By-products provide even more compounds that could inhibit the enzyme of interest. We will address this again shortly.

By-products may also be formed as a result of further metabolism of your desired product. In our indene example, an EE upgrade was observed from 30-40% initially to >98% in a kinetic manner, *ie*, over time following the initial conversion. This was tied to a drop in the total product concentration, the result of an enzyme selectively converting one enantiomer to another end-product. Thus, you need to be aware of whether or not your product is another enzyme's substrate! Again, the most important aspect of by-product formation is to recognize that it exists. Then, you can look for solutions to the problem, as we'll discuss in the next set of lectures.

C. Substrate/Product Inhbition/Toxicity

We will talk about design strategies around substrate/product toxicity in the next set of lectures. What's important to note here is that you must be *aware* of the possibility of substrate and or product inhibition, and you need to know how to look for it. One clue that an enzyme may be suffering from inhibition/toxicity is if the reaction fails to go towards high conversion, even as you increase the amount of catalyst (cells) that are present. This would then suggest that there's not a limitation in the turnover of the enzyme, but that the enzyme is being negatively impacted by something in the reaction. In whole cell reactions, you must also remember that you have other compounds in the spent medium and possibly many by-products that could inhibit the enzyme of interest. Look again at the indene example. In the original *Pseudomonas* culture, inhibition was observed with three different metabolites! If we know that inhibition exists, we can look for ways (engineering or biological solutions) to overcome it.

3. Putting it all together…

Once you have determined the best growth conditions for your organism (and remember that we've left out the area of medium optimization), and the best operating conditions for the conversion, you can determine the best specific activity of your whole cell catalyst and the amount of biomass you'll need to perform the conversion. Based on the standard exponential growth behavior, you can get an estimate of the cycle time for growth. Keep

in mind, though, that you will need to experimentally determine whether or not there is a lag phase, and if (as in the case of catabolite repression) you need to let the cells go past the exponential phase and into the stationary phase to get highest activity. One "easy" aspect of whole cell design is that the catalyst can be treated like a purified enzyme for the purpose of determining kinetics. After all, there's still an enzyme performing the conversion, so you are still likely to observe Michalis-Menten form kinetics. The specific activity is then simply a measure of the reaction rate at a particular substrate concentration. You can then determine the conversion time based on the kinetic profile (or the specific activity) and the desired amount of substrate to be converted. Combining these two together will then give you a cycle time for the whole cell bioconversion.

In the next set of lectures, we'll talk about engineering solutions to process problems that may arise for both free enzyme and whole cell processes.