# CINÉTICA E REGULAÇÃO ENZIMÁTICA | DESIGN EXPERIMENTAL EM ENZIMOLOGIA

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- Experimental design includes both
  - Strategies for organizing data collection
  - Data analysis procedures matched to those data collection strategies
- Classical treatments of design stress analysis procedures based on statisitcal analysis
- Other analysis procedure such as those based on hierarchical linear models or analysis of aggregates (e.g., class or school means) are also appropriate

# Why we need Experimental Design?

- Because of variability

- We wouldn't need a science of experimental design if
  - If all units (students, teachers, & schools) were identical

and

  - If all units responded identically to treatments

- We need experimental design to control variability so that treatment effects can be identified

- The idea of controlling variability through design has a long history

- In 1747 Sir James Lind's studies of scurvy

- Their cases were as similar as I could have them. They all in general had putrid gums, spots and lassitude, with weakness of their knees. They lay together on one place … and had one diet common to all (Lind, 1753, p. 149)

- Lind then assigned six different treatments to groups of patients

- The idea of random assignment was not obvious and took time to catch on
- In 1648 von Helmont carried out one randomization in a trial of bloodletting for fevers
- In 1904 Karl Pearson suggested matching and alternation in typhoid trials
- Amberson, et al. (1931) carried out a trial with one randomization
- In 1937 Sir Bradford Hill advocated alternation of patients in trials rather than randomization
- Diehl, et al. (1938) carried out a trial that is sometimes referred to as randomized, but it actually used alternation

- Studies in crop variation I – VI (1921 – 1929)
- In 1919 a statistician named Fisher was hired at Rothamsted agricultural station
- They had a lot of observational data on crop yields and hoped a statistician could analyze it to find effects of various treatments
- All he had to do was sort out the effects of confounding variables

- Fisher does regression analyses—lots of them—to study (and get rid of) the effects of confounders
  - soil fertility gradients
  - drainage
  - effects of rainfall
  - effects of temperature and weather, etc.

- Fisher does qualitative work to sort out anomalies

- Conclusion: The effects of confounders are typically larger than those of the systematic effects we want to study

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**HOW TO TRANSLATE THIS TO ENZYMOLOGY**

?

- Fisher does qualitative work to sort out anomalies
- Conclusion: The effects of confounders are typically larger than those of the systematic effects we want to study

- Fisher invents

- Basic principles of experimental design
- Control of variation by randomization
- Analysis of variance

- Studies in Crop variation IV (1927)
  - Fisher invents analysis of covariance to combine statistical control and control by randomization

- Studies in crop variation VI (1929)
  - Fisher refines the theory of experimental design, introducing most other key concepts known today

- Experimental design controls background variability so that systematic effects of treatments can be observed

- Three basic principles
  - Control by matching
  - Control by randomization
  - Control by statistical adjustment

- Their importance is in that order

**WHAT ARE TREATMENTS IN ENZYME REGULATION**

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- Known sources of variation may be eliminated by matching
- Eliminating genetic variation
  - Compare animals from the same litter of mice
- Eliminating district or school effects
  - Compare students within districts or schools
- However matching is limited
  - matching is only possible on observable characteristics
  - perfect matching is not always possible
  - matching inherently limits generalizability by removing (possibly desired) variation
- Matching ensures that groups compared are alike on specific known and observable characteristics (in principle, everything we have thought of)
- Wouldn't it be great if there were a method of making groups alike on not only everything we have thought of, but everything we didn't think of too?

**CONSIDERING AN ENZYME PRODUCED BY A WILD MICRORGANISM AND WE WNAT TO STUDY ITS REGULATION BY TEMPERATURE, HOW MATCHING COULD BE CONTROLED?**

?

# Control by Randomization

- Matching controls for the effects of variation due to specific observable characteristics
- Randomization controls for the effects all (observable or non-observable, known or unknown) characteristics
- Randomization makes groups equivalent (on average) on all variables (known and unknown, observable or not)
- Randomization also gives us a way to assess whether differences after treatment are larger than would be expected due to chance.
- Random assignment is not assignment with no particular rule. It is a purposeful process
- Assignment is made at random. This does not mean that the experimenter writes down the names of the varieties in any order that occurs to him, but that he carries out a physical experimental process of randomization, using means which shall ensure that each variety will have an equal chance of being tested on any particular plot of ground (Fisher, 1935, p. 51)

**CONSIDERING NA ENZYME E1 REGULATED BY 3 INHIBITORS A, B AND C THAT YOU WANT TO STUDY IN A SINGLE CONCENTRATION (3 REPLICATES). HOW COULD BE A RANDOM DESIGN FOR THIS**

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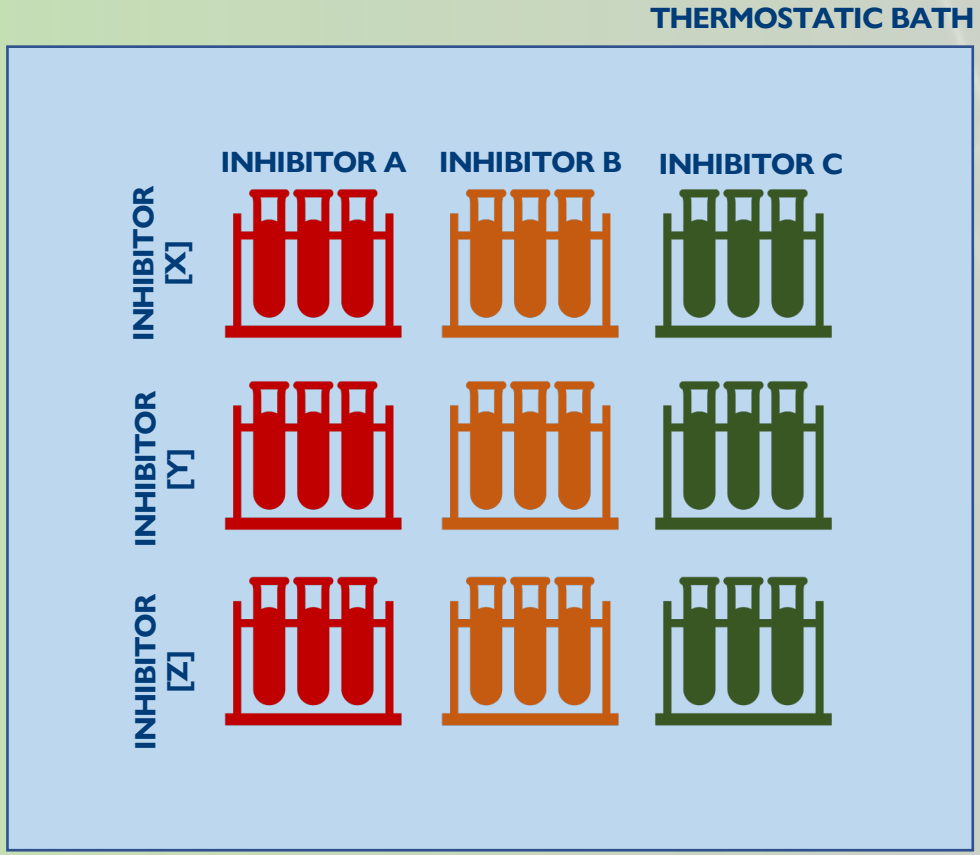


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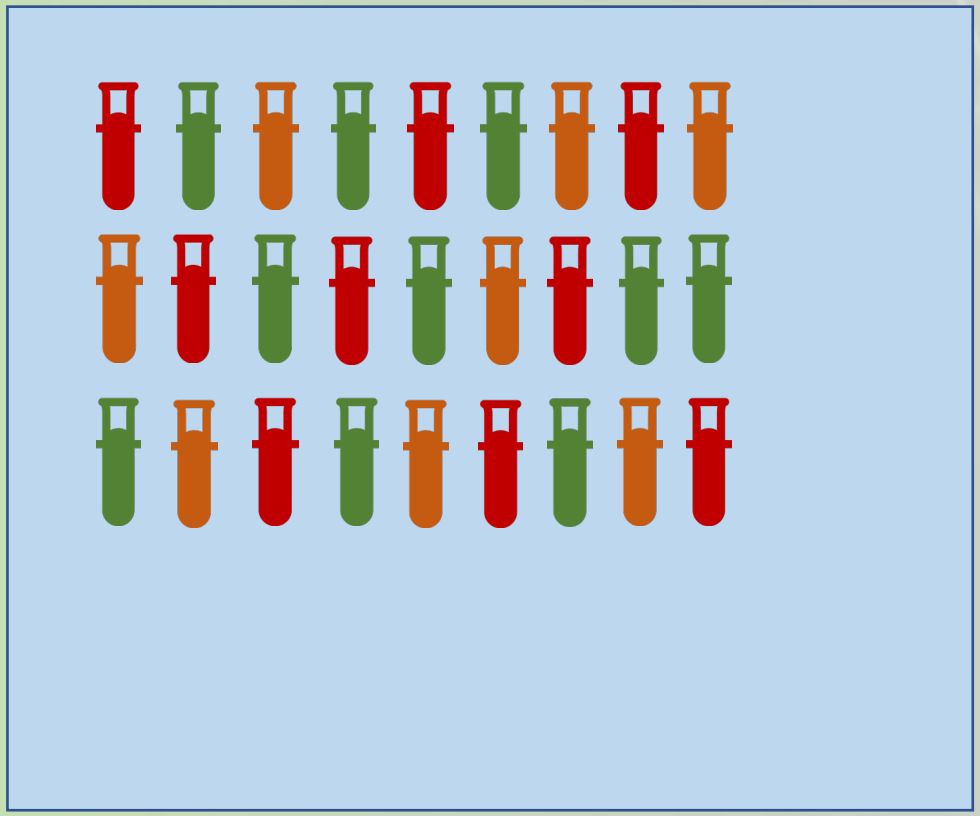

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# Control by Statistical Adjustment

- The use of control variables for statistical adjustment is motivated primarily by a desire to increase the internal validity of the study
- Control by statistical adjustment is a form of pseudo-matching
- It uses statistical relations to simulate matching
- Statistical control is important for increasing precision but should not be relied upon to control biases that may exist prior to assignment

**CONSIDERING THE PREVIOUS EXAMPLE, WHERE IS THE STATISTICAL CONTROL**

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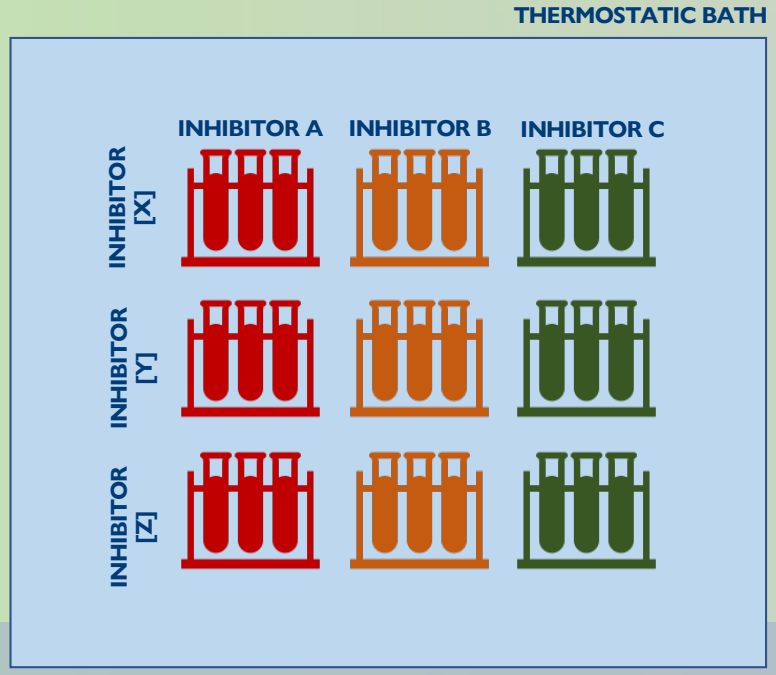


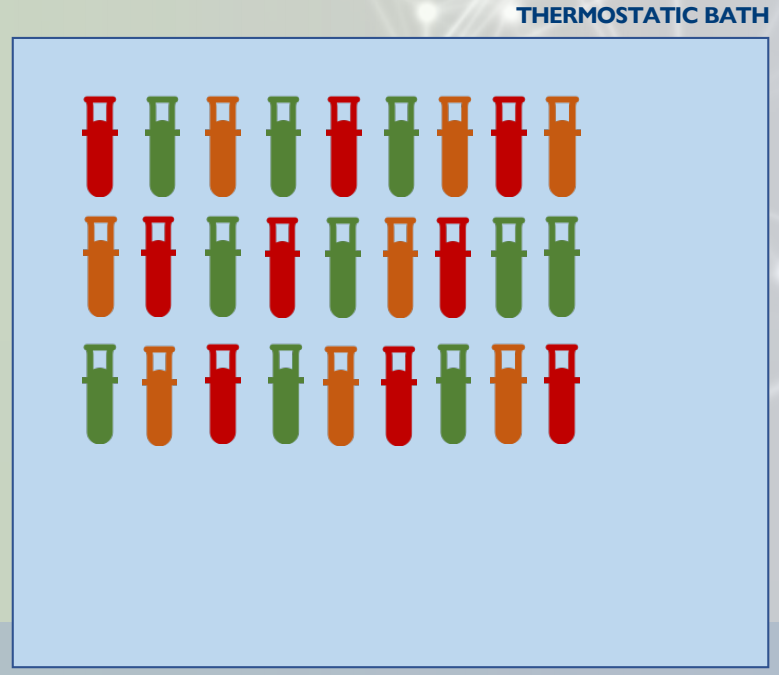

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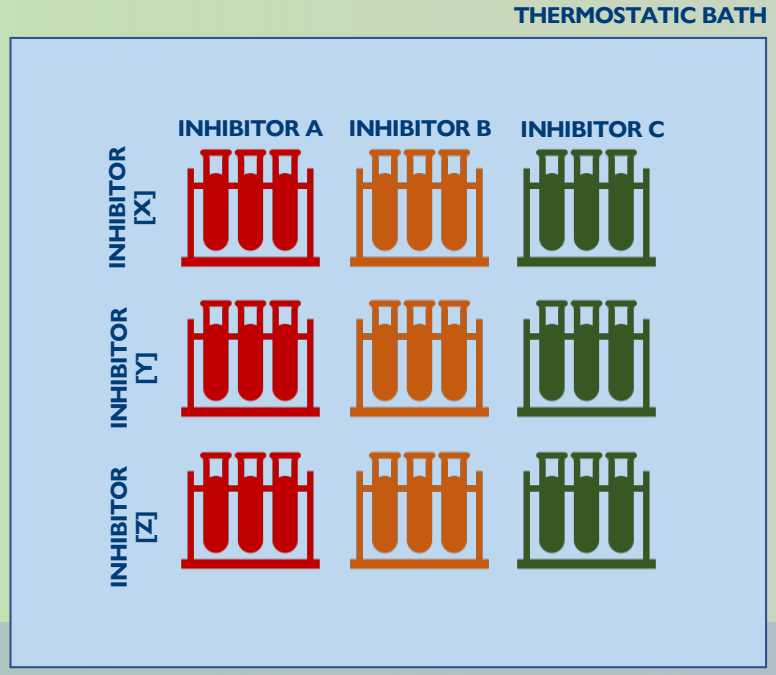


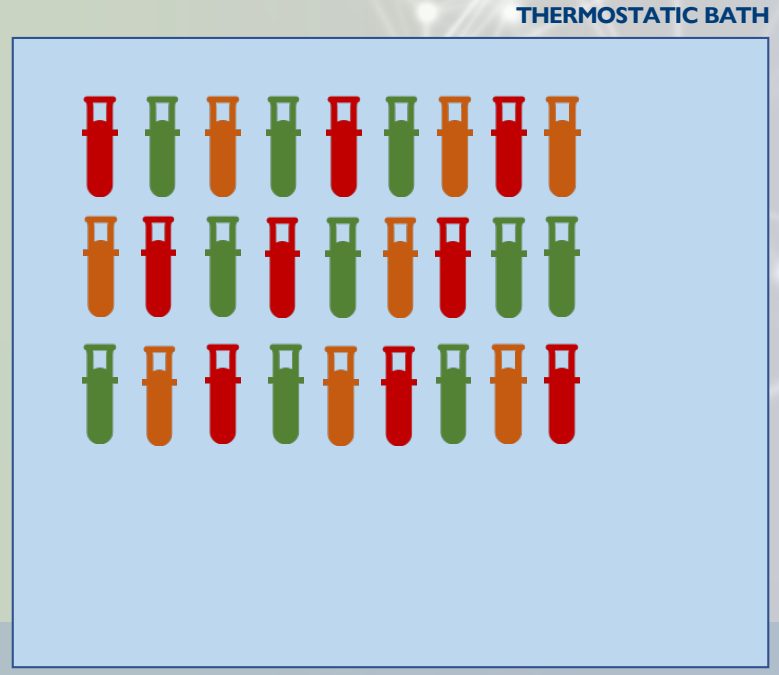

- You have to know a lot (be smart) to use matching and statistical control effectively
- You do not have to be smart to use randomization effectively
- But
- Where all are possible, randomization is not as efficient (requires larger sample sizes for the same power) as matching or statistical control

# Basic Ideas of Design: Independent Variables (Factors)

- The values of independent variables are called levels
- Some independent variables can be manipulated, others can't
- Treatments are independent variables that can be manipulated
- Blocks and covariates are independent variables that cannot be manipulated
- These concepts are simple, but are often confused
- Remember:
- You can randomly assign treatment levels but not blocks

**GIVE ME EXAMPLES OF TREATMENTS AND OF BLOCKS**

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**EXAMPLE:**

**AN ENZYME IS REGULATED BY TEMPERATURE AND ALSO BY ITS REACTION PRODUCT.**

**TEMPERATURE CAN BE REGULATED BUT WILL INEVITABLY AFFECT THE [P] THAT COVARIATE WITH TEMPERATURE AND ENZYME ACTIVITY**

- Relations between independent variables
- Factors (treatments or blocks) are crossed if every level of one factor occurs with every level of another factor
- Example
- The Tennessee class size experiment assigned students to one of three class size conditions. All three treatment conditions occurred within each of the participating schools
- Thus treatment was crossed with schools

**HOW CROSSING CAN INCORPORATE THE EFFECT OF THE PRODUCED SUBSTRATE**

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**HOW CROSSING CAN INCORPORATE THE EFFECT OF THE PRODUCT**

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**PREFORME INCUBATIONS AT ΔT AND Δ[P] WHERE [P]i IS USED AS BLANK**

- Factor B is nested in factor A if every level of factor B occurs within only one level of factor A

- Example
  - The Tennessee class size experiment actually assigned classrooms to one of three class size conditions. Each classroom occurred in only one treatment condition
  - Thus classrooms were nested within treatments
  - (But treatment was crossed with schools)

**WHAT IS NESTED IN WHAT?**

**NA ENZYME IS REGULATED BY PH. IT'S SUBSTRATE IS AN ACID.**

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**WHAT IS NESTED IN WHAT?**

**NA ENZYME IS REGULATED BY PH. IT'S SUBSTRATE IS AN ACID.**

**[S] IS NESTED IN PH AS EACH [S] WILL CHANGE PH TO A CERTAIN UNIQUE VALUE IF ALL REMAINS CONSTANT**

# Three Basic Designs

- The completely randomized design
- Treatments are assigned to individuals

- The randomized block design
- Treatments are assigned to individuals within blocks

- The hierarchical design
- Treatments are assigned to blocks, the same treatment is assigned to all individuals in the block

# Three Basic Designs

- **The completely randomized design**
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- **The hierarchical design**
- **Treatments are assigned to blocks, the same treatment is assigned to all individuals in the block**

## The Completely Randomized Design

Individuals are randomly assigned to one of two treatments

| Treatment | Control |
|---|---|
| Individual 1 | Individual 1 |
| Individual 2 | Individual 2 |
| ⋮ | ⋮ |
| Individual *n* | Individual *n* |

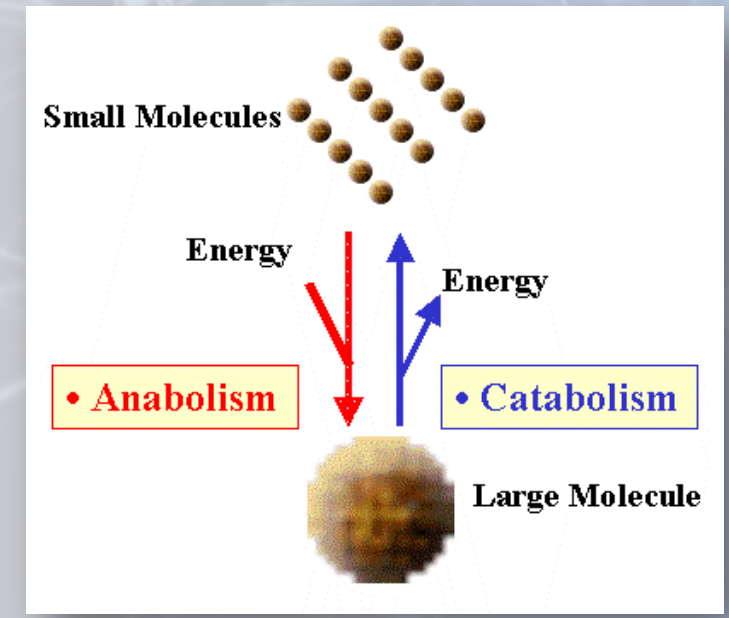
## The Randomized Block Design

| | Block 1 | … | Block *m* |
|---|---|---|---|
| Treatment 1 | Individual 1<br>⋮<br>Individual *n* | … | Individual 1<br>⋮<br>Individual *n* |
| Treatment 2 | Individual *n* +1<br>⋮<br>Individual 2*n* | … | Individual *n*+1<br>⋮<br>Individual 2*n* |

## The Hierarchical Design

| Treatment | | Control | |
|---|---|---|---|
| Block 1 | Block *m* | Block *m+1* | Block 2*m* |
| Individual 1 | Individual 1 | Individual 1 | Individual 1 |
| Individual 2 | Individual 2 | Individual 2 | Individual 2 |
| ⋮ … | ⋮ | ⋮ … | ⋮ |
| Individual *n* | Individual *n* | Individual *n* | Individual *n* |

- **Life is dependent on chemical reactions.**
  - Life is dependent on both the formation of different molecules into new molecules (anabolism) and on the breaking of one molecule into multiple molecules (catabolism).
  - For example, all living things depend on plants to combine carbon dioxide and water into sugar during photosynthesis.
  - All living things break down sugar into carbon dioxide and water, and use this process to generate ATP in order to power cellular activity.
- **Chemical reactions rarely 'just happen'.**
  - Most molecules do not just combine with each other. Most molecules do just break apart automatically.
  - The majority of chemical reactions requires an input of energy and often involves specific conditions and circumstances in order to occur.



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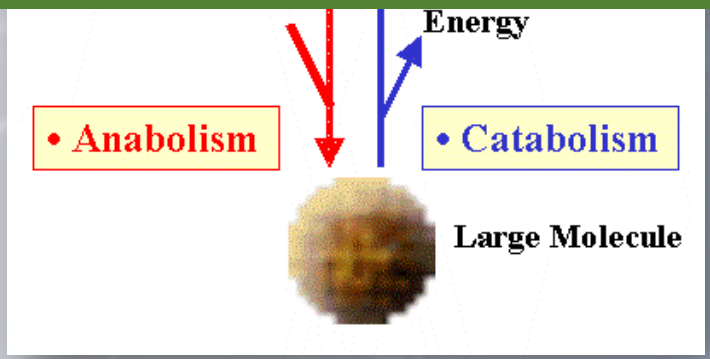
**First step in Experimental Designing in Enzymology**

**IDENTIFY A REACTION OF INTEREST.**

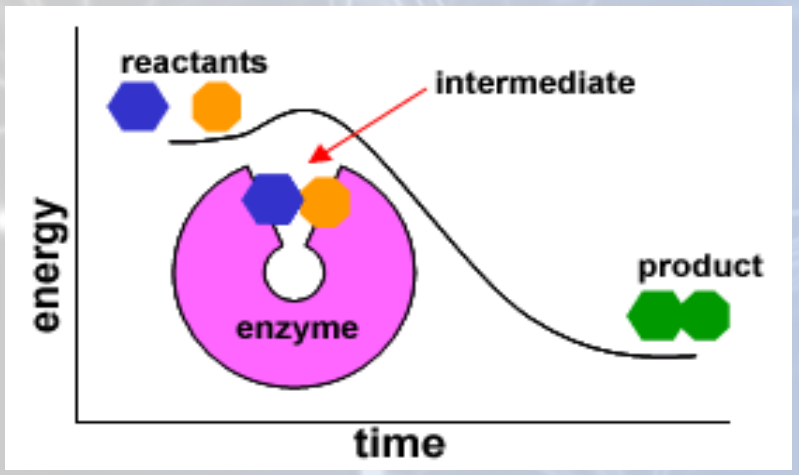
**WHY IS IT IMPORTANT? IS IT WORTH THE INVESTEMENT?**

**WHY IT IS IMPORTANT TO STUDY AN ALTERNATIVE TO THE CHEMICAL PROCESSES?**



- **In a chemical reaction, the molecules at the start of a reaction are called the reactants.**
  - The molecules formed as a result of the reaction are called products.
  - The energy needed to start a reaction is called the activation energy.

- **Enzymes are protein catalysts that lower the activation energy of a reaction in order to increase the rate of the formation of a product in a biochemical reaction.**
  - Enzymes are needed by living organisms because they increase the rate at which biochemical reactions necessary for life can occur.
  - Without enzymes, the reactions needed for living organisms to function could not occur at a fast enough rate.



- **Enzymes are catalysts, or chemicals that enable a reaction to occur more easily because they lower the activation energy of a reaction.**
  - A certain amount of energy is needed when two molecules react with each other in order to form a product.
  - If the two molecules that react in a chemical reaction do not contain a sufficient amount of energy, then the reaction cannot occur and no product will be formed.

- **Enzymes and other kinds of catalysts lower the amount of energy that is needed for a reaction to occur and for a product to be formed from a substrate without being a part of the final product formed in a reaction.**
  - A substrate is a reactant in an enzymatic reaction that an enzyme acts upon to create a product.
  - The product is the molecule created as a result of a chemical reaction.

- **Enzymes are <u>catalysts</u>, or chemicals that enable a reaction to occur more easily because they lower the activation energy of a reaction.**
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  - A <u>substrate</u> is a reactant in an enzymatic reaction that an enzyme acts upon to create a product.
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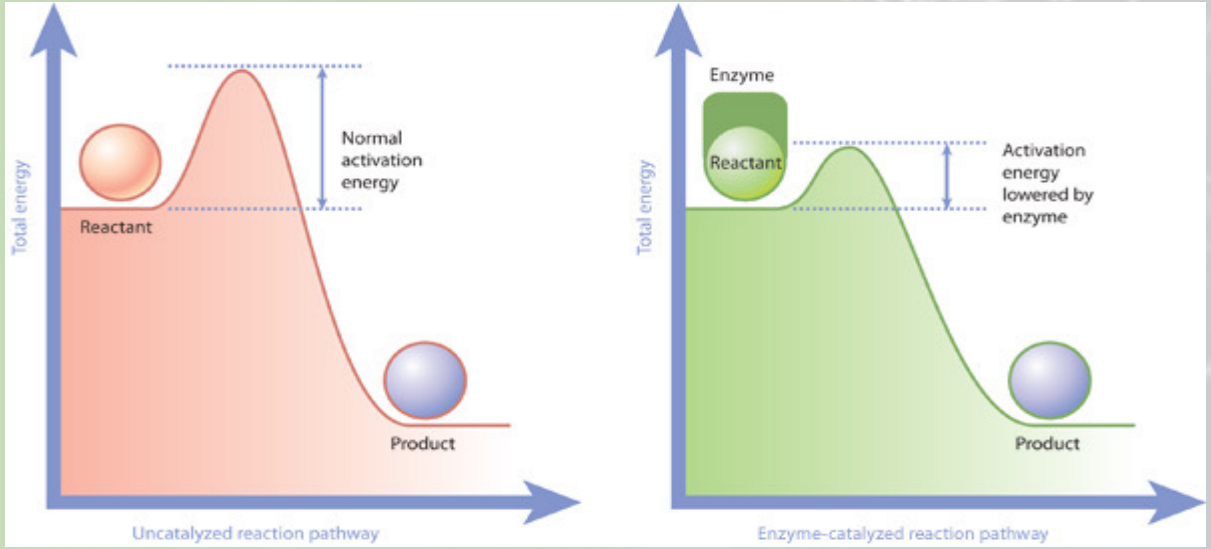
**Second step in Experimental Designing in Enzymology**

**IS THERE AN ENZYME THAT CATALYSES THE REACTION OF INTEREST?**

**WAS IT FOUND? WHERE? WHEN? IN WHICH ORGANISM?**

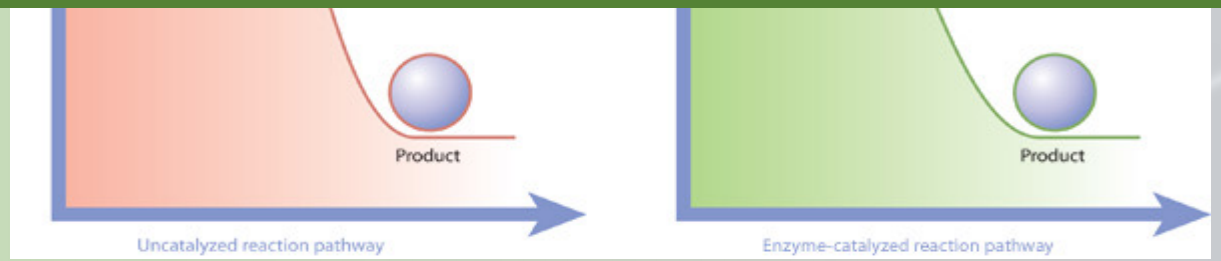
- **Chemical reactions are sort of like a car going up a mountain.**
  - If the car does not have enough gas to get up the mountain, it will roll back to its starting point.
  - Enzymes in this analogy wouldn't increase the amount of gas in the car; instead, they would lower the height of the mountain.
  - In this case, enzymes would be like the excavation equipment that lower the height of the hills so that less energy is needed to reach the top of each hill.



- **Chemical reactions are sort of like a car going up a mountain.**
  - If the car does not have enough gas to get up the mountain, it will roll back to its starting point.
  - **Third step in Experimental Designing in Enzymology**
  - **WHAT ARE THE REGULATION FACTORS OF THE ENZYME?**

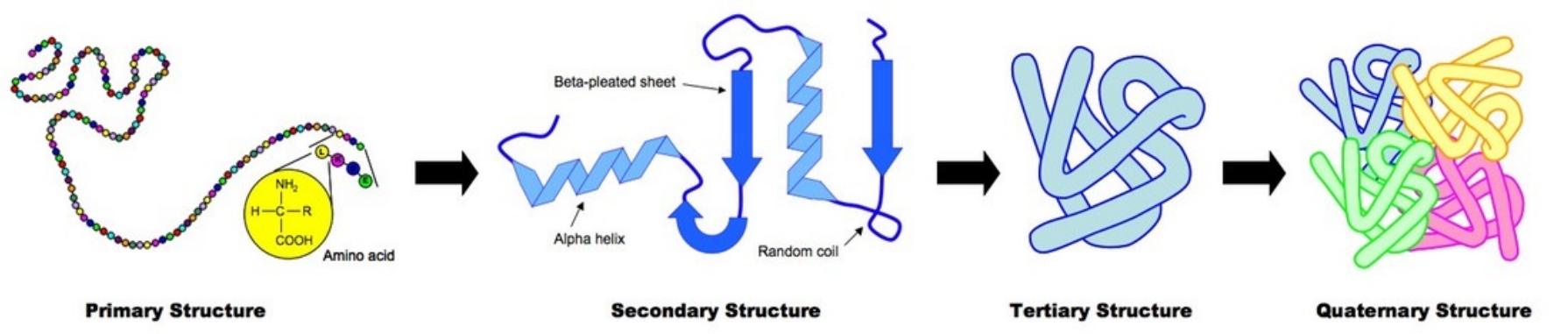
    **WHAT ARE THE RANGES?**

    **ARE THESE REGULATORY FACTORS AND OPTIMUM RANGES OF ACTIVITY OF ADDED-VALUE FOR THE INDUSTRY?**



- **Enzymes are proteins, or nitrogen-based biological macromolecules that are formed through specific combinations of amino acids.**
  - A macromolecule is a molecule that contains a very large number of molecules.

- **Proteins are essential to the function and existence of every living organism.**
  - In addition to enzymes that lower the activation energy of biochemical reactions, proteins can also work as pumps, serve structural roles, manufacture other molecules (such as ATP), fight pathogens (e.g. antibodies), send messages (e.g. hormones), and play many other roles.
  - Proteins are often the *molecular machines* of an organism.



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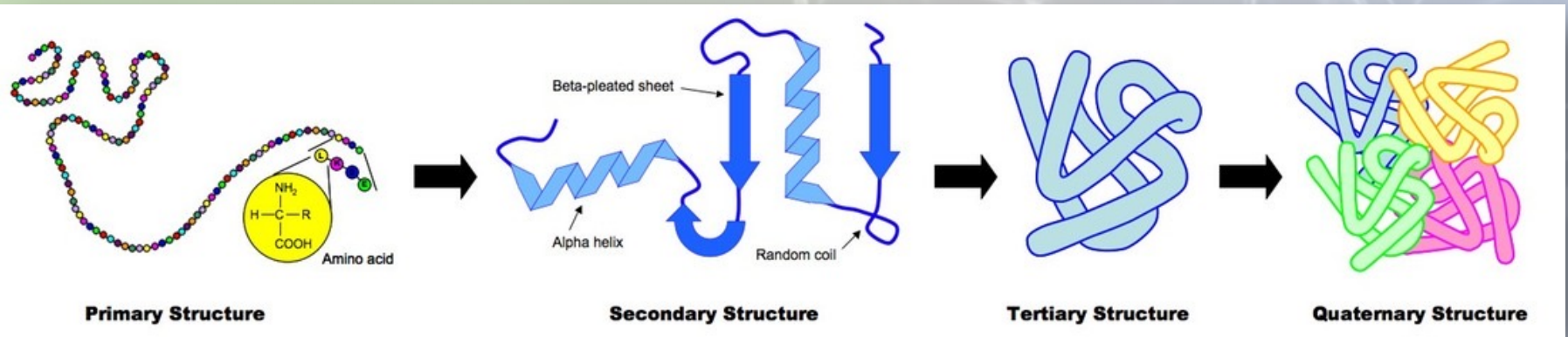
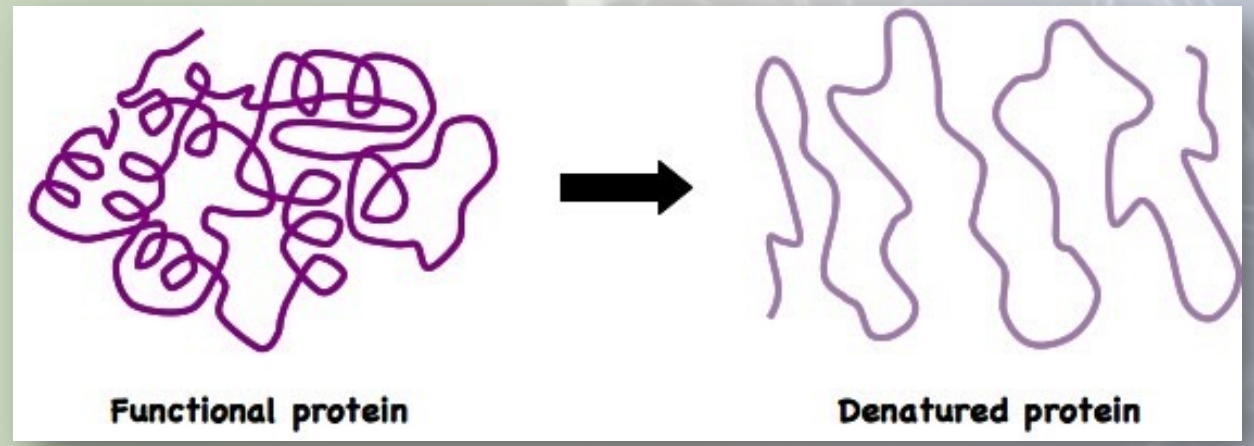
**Fourth step in Experimental Designing in Enzymology**

**WHAT ARE THE NON-OPTIMAL CONDITIONS FOR THE ENZYME FUNTIONING?**



- **Temperature, pH, and concentration are also vital to the function of an enzyme.**
  - The rate of enzyme activity usually doubles with every increase of 10 degrees due to the fact that molecules involved in the reaction will move faster and collide more often as the temperature increases.
  - However, if temperatures get too high, the enzymes will denature (unfold and lose their shape).
  - This is why a fever becomes dangerous for a human being when it gets above 104° F.



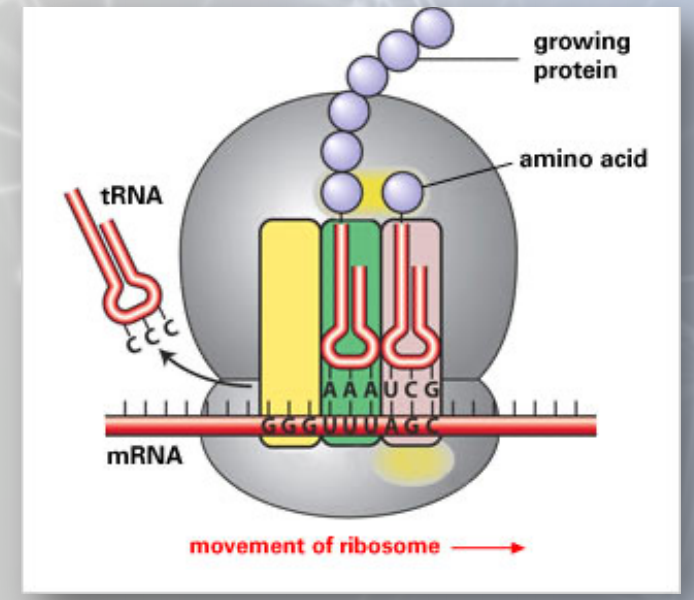
- **Enzymes also are dependent on a specific pH to function.**
  - If a solution becomes too acidic or basic, an enzyme will become less functional and may even denature under extreme changes to a pH.

- **Enzymes also depend on a sufficient concentration of both the enzyme and the substrate in order to function.**
  - This is because of the fact that enzymes and substrates must make physical contact in order to function.
  - As long as there are enzymes with open active sites, an increase in the amount of substrate will increase the amount of enzyme activity.

- **Proteins are formed through transcription and translation.**
  - Transcription is when DNA is copied by mRNA.
  - Translation is when a ribosome makes a protein using amino acids delivered by tRNA based on the information copied from DNA by mRNA.

- **As amino acids are delivered to the ribosome, they are assembled into a chain (sort of like pearls on a necklace).**
  - This chain will begin to fold into a specific shape based on the chemical properties of the amino acids.
  - The shape in which the protein folds will determine the function of the protein.

- **Shape is very important for enzymes.**
  - Enzymes usually work by providing a specifically-shaped active site in which a specific molecule binds.



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**Fifth step in Experimental Designing in Enzymology**

**WHERE IS IT CODED?**

**HOW MANY GENES AND ISOFORMS?**

**IS RECOMBINANT TECHNOLOGY RELIEABLE AND MORE THAN ANYTHING ELSE NEEDED VERSUS THE USE OF THE ORIGINAL SOURCE?**

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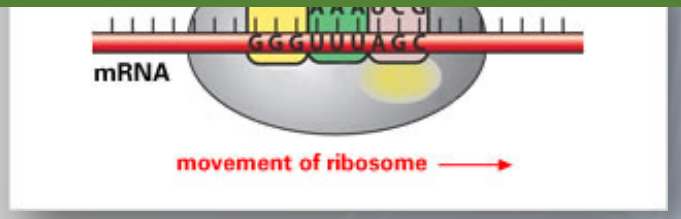

- **Living organisms may provide insights as to how enzymes can be acquired, utilized, and improved in order to make it more feasible and cost effective to produce the product of interest.**
  - Bioprospecting is the process of searching for species in nature that can provide valuable products for human purposes.
  - These products can include chemical compounds, genes, microorganisms, and more.

- **EXAMPLE: The research related to the *T. reesei* fungus provided the insight as to the mechanism in which cellulase enzymes were able to break down cellulose into glucose.**
  - This research represented early work in the field of bioprospecting because it enabled researchers to not only understand the mechanism of these enzymes but also develop industrial strains of this fungus in order to produce larger quantities of the needed enzymes.

- Bioprospecting has also been valuable for agriculture.
  - *For example, Bt Corn is a widely-utilized genetically modified crop that has a gene so that it can produce a naturally-occurring insecticide from a species of bacteria.*
  - *Because Bt Corn produces its own insecticide, it significantly reduces the need for spayed applications of synthetic pesticides and greatly reduces the harmful impact on beneficial pollinating insects.*

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**How is this related to Enzymology?**

**Because the expression of this insecticide is only of added-value because the target insects don't have enzymes to metabolize this biocide molecule.**

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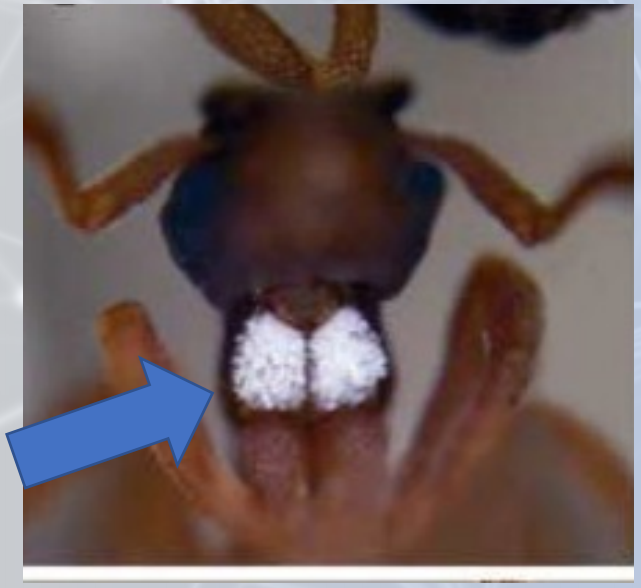
- **Bioprospecting for more effective versions of cellulase enzymes has broadened far beyond the initial research of *T. reesei*.**
  - One successful modern example of bioprospecting for cellulase enzymes involves the leaf cutter ant, *Atta colubmica*.
  - This leaf cutter ant has developed a symbiotic relationship with a funcus called *L. gonglyophorus*.
  - This fungus only grows in the nests of this particular species of leaf cutter ants by breaking down the leaves that are brought to it by the ants themselves.

- **The leaf cutter ants and fungus that they feed are evidence that complex organisms (like ants and humans) can use a cellulosic source of fuel to meet all of their energy needs.**
  - For 50 million years, these ants have fed cellulosic feedstocks to this fungus and in exchange the fungus provides a mixture of lipids, proteins, and carbohydrates that feed the ants.
  - This fungus only uses the hemicellulose, leaving behind lignin and cellulose waste that the ants remove and deposit in a 'dump' in their colony.
  - Additional forms of fungi and bacteria further degrade this lignin and cellulose.

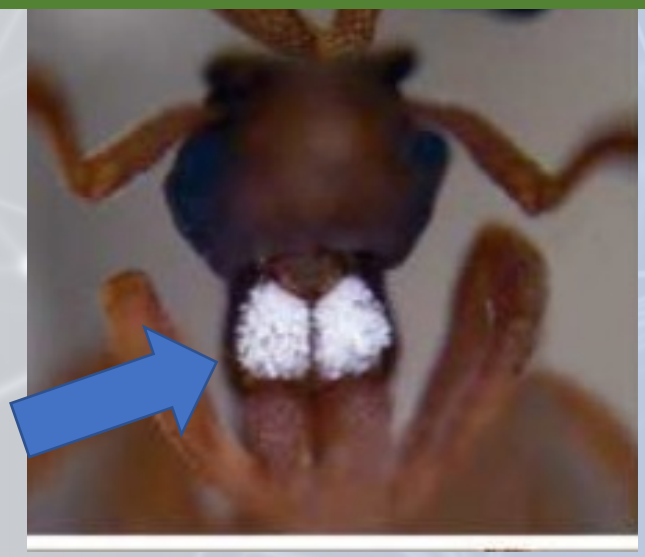
- **Furthermore, the ants have specialized-bacteria that living on their bodies that produce an antibiotic that the ants use to protect the fungus.**
  - The ants have been using these antibiotics for 50 million years with the development of antibiotic resistance (a phenomenon in which an antibiotic loses its effectiveness over time due to overuse).
  - The fact that humans have only been using antibiotics for several decades and have already had to stop using some because of antibiotic resistance is evidence for the fact that these ant colonies and their symbiotic relationships may yield large amounts of valuable information.
  - The enzymes that the fungi use to break down the mulched leaves, the bacteria in the dumps of the ants, and the antibiotics used by the ants may all be useful for human purposes in bioenergy, medicine, agriculture, and much more.



- **Furthermore, the ants have specialized-bacteria that living on their bodies that produce an antibiotic that the ants use to protect the fungus.**
  - The ants have been using these antibiotics for 50 million years with the development of antibiotic resistance (a phenomenon in which an antibiotic loses its effectiveness over time due
  - 
    large amounts of valuable information.
  - The enzymes that the fungi use to break down the mulched leaves, the bacteria in the dumps of the ants, and the antibiotics used by the ants may all be useful for human purposes in bioenergy, medicine, agriculture, and much more.

**IMAGINE YOU WANT TO STUDY THIS ENZYME FROM A FUNGUS THAT LIVES IN THE ANT'S NESTS?**

**First approaches?**



*Bioprospecting generally consists of five phases.*

- **<u>Phase 1</u>: determination of environmental characteristics necessary to support and select for traits sought in target organisms and formation of hypotheses related to where to search for organisms.**
  - For example, if you were seeking cellulase-producing microbes and fungi, you would need an environment that both ***enables*** the growth of these organisms (wet and warm) while also ***selecting*** for organisms that produce cellulase over other types of organisms (which might occur if cellulose were the only source of energy available in that environment).

- **<u>Phase 2</u>: on-site collection of samples in areas hypothesized to be supportive and selective for the target organisms.**

- **Phase 3: culturing, isolation, and identification of target organisms.**
- **Phase 4: screening of the target organism(s) for their ability to produce the needed compounds.**
- **Phase 5: development and industrial production of the acquired compounds.**
  - This could be developed through selective breeding of the target organism to improve its ability to produce the needed compounds.
  - This could also occur through identification of the genes associated with the needed compounds and insertion of these genes into other organisms that are easier to grow in a laboratory, agricultural, or industrial setting.
  - It may also be possible to artificially produce the compound without the use of the organism (as is the case with the acetylsalicylic acid in aspirin).