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Module I 8L

Lecture 1

Introduction to Enzyme Technology

1. Introduction:

In enzyme technology – a subfield of biotechnology – new processes have been and are being developed to manufacture both bulk and high added-value products utilizing enzymes as biocatalysts, in order to meet needs such as food (e.g., bread, cheese, beer, vinegar), fine chemicals (e.g., amino acids, vitamins), and pharmaceuticals. Enzymes are also used to provide services, as in washing and environmental processes, or for analytical and diagnostic purposes. The driving force in the development of enzyme technology, both in academia and in industry, has been and will continue to be

- the development of new and better products, processes, and services to meet these needs, and/or
- the improvement of processes to produce existing products from new raw materials such as biomass.

Differences between enzymes and chemical catalysts:

Enzymes and **catalysts** both affect the rate of a reaction. In fact, all known enzymes are catalysts, but not all catalysts are enzymes. The **difference between catalysts and enzymes** is that enzymes are largely organic in nature and are bio-catalysts, while non-enzymatic catalysts can be inorganic compounds. Neither catalysts nor enzymes are consumed in the reactions they catalyze.

	Catalyst	**Enzyme**
Function	Catalysts are substances that increase or decrease the rate of a chemical reaction but remain unchanged.	Enzymes are proteins that increase rate of chemical reactions converting substrate into product.
Molecular weight	Low molecular weight compounds.	High molecular weight globular proteins.
Types	There are two types of catalysts – positive and negative catalysts.	There are two types of enzymes - activation enzymes and inhibitory enzymes.
Nature	Catalysts are simple inorganic	Enzymes are complex

	Catalyst	Enzyme
	molecules.	proteins.
Alternate terms	Inorganic catalyst.	Organic catalyst or bio catalyst.
Reaction rates	Typically slower	Several times faster
Specificity	They are not specific and therefore end up producing residues with errors	Enzymes are highly specific producing large amount of good residues
Conditions	High temp, pressure	Mild conditions, physiological pH and temperature
C-C and C-H bonds	absent	present
Example	vanadium oxide	amylase, lipase
Activation Energy	Lowers it	Lowers it

Lecture 2:

Classification, Nomenclature, and Examples of Enzymes:

1.1. Classification, Nomenclature, and Examples of Enzymes:

- **Oxidoreductases**

These catalyze oxidation and reduction reactions,e.g. pyruvate dehydrogenase, which catalyzes the oxidation of pyruvate to acetyl coenzyme A.

- **Transferases**

These catalyze the transfer of a chemical group from one compound to another. An example is a transaminase, which transfers an amine group from one molecule to another.

- **Hydrolases**

They catalyze the hydrolysis of a bond. For example, the enzyme pepsin hydrolyzes peptide bonds in proteins.

- **Lyases**

These catalyze breakage of bonds without catalysis, e.g. aldolase (an enzyme in glycolysis) catalyzes the splitting of fructose-1, 6-bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate.

- **Isomerases**

They catalyze the formation of an isomer of a compound, example,phosphoglucomutase catalyzes the conversion of glucose-1-phosphate to glucose-6-phosphate (transfer of a phosphate group from one position to another in the same compound) in glycogenolysis (conversion of glycogen to glucose for quick release of energy.

- **Ligases**

Ligases catalyze the joining of two molecules. For example, DNA ligase catalyzes the joining of two fragments of DNA by forming a phosphodiester bond.

Cofactors:

Co-factors are non-proteinous substances that associate with enzymes. A cofactor is essential for the functioning of an enzyme. An enzyme without a cofactor is called an apoenzyme. An apoenzyme and its cofactor together constitute the holoenzyme.

There are three kinds of cofactors present in enzymes:

- **Prosthetic groups**: These are cofactors tightly bound to an enzyme at all times. A fad is a prosthetic group present in many enzymes.

- **Coenzyme**: A coenzyme is bound to an enzyme only during catalysis. At all other times, it is detached from the enzyme. NAD^+ is a common coenzyme.

- **Metal ions**: For the catalysis of certain enzymes, a metal ion is required at the active site to form coordinate bonds. Zn^{2+} is a metal ion cofactor used by a number of enzymes.

Lecture 3:

Industrial Enzymes – Present Status and Opportunities with Special Reference to Food industries

1.2. Industrial Enzymes – Present Status and Opportunities with Special Reference to Food industries:

Enzymes play a vital role in food industry

- Cheese and brewing rely on enzyme

- activity in various stages of processing Trial and error been able to optimize
- conditions –malting, resting animals prior to slaughter Traditional products like yoghurt depend
- on enzymes –but whole organisms
- Whole organisms give characteristic notes in the product that can not be achieved by purified enzymes
- Enzymes used may be endogenous like in amylase in mashing, or in yoghurt

Accessibility of substrate by enzymes

- Some enzymes found free in cytoplasm but many are bound to membrane and almost in contact with substrate
- If exogenous enzymes are to be used = cross the membrane barrier. Intact membranes are impermeable to large molecules like exogenous enzymes
- Tenderization of meat – not able to effect CT unless during cooking, penetration – dips, injections prior to slaughter

Reaction conditions

- Enzyme reactions occur not under idealconditions (temperature, substrate, pH) . It is difficult to predict the amount of enzyme required
- Substrate concentration is another problem of applying biochemical criteria
- commercial enzymes operate at 50-100oC as opposed to 25oC

Physical factors affect enzyme action,

- reaction rates in solution and those that are bound to membranes Sources of enzymes Most organisms have certain core
- enzymes – Embden Meyerhof pathway, amylase in human saliva and seeds potential source

Lecture 4:

Sources of Enzymes

1.3. Sources of Enzymes:

Biologically active enzymes may be extracted from any living organism. A very wide range of sources are used for commercial enzyme production from *Actinoplanes* to *Zymomonas*, from spinach to snake venom. Of the hundred or so enzymes being used industrially, over a half are from fungi and yeast and over a third are from bacteria with the remainder divided between animal (8%) and plant (4%) sources. A very much larger number of enzymes find use in chemical analysis and clinical diagnosis. Non-microbial sources provide a larger proportion of these, at the present time. Microbes are preferred to plants and animals as sources of enzymes because:

i. they are generally cheaper to produce.
ii. their enzyme contents are more predictable and controllable,
iii. reliable supplies of raw material of constant composition are more easily arranged, and
iv. plant and animal tissues contain more potentially harmful materials than microbes, including phenolic compounds (from plants), endogenous enzyme inhibitors and proteases.

Attempts are being made to overcome some of these difficulties by the use of animal and plant cell culture.

Some important industrial enzymes and their sources:

Enzyme[a]	EC number[b]	Source	Intra/extra -cellular[c]	Scale of production[d]	Industrial use
Animal enzymes					
Catalase	1.11.1.6	Liver	I	-	Food
Chymotrypsin	3.4.21.1	Pancreas	E	-	Leather
Lipase[e]	3.1.1.3	Pancreas	E	-	Food
Rennet[f]	3.4.23.4	Abomasum	E	+	Cheese
Trypsin	3.4.21.4	Pancreas	E	-	Leather
Plant enzymes					
Actinidin	3.4.22.14	Kiwi fruit	E	-	Food
a-Amylase	3.2.1.1	Malted barley	E	+++	Brewing
b-Amylase	3.2.1.2	Malted barley	E	+++	Brewing
Bromelain	3.4.22.4	Pineapple latex	E	-	Brewing
b-Glucanase[a]	3.2.1.6	Malted barley	E	++	Brewing
Ficin	3.4.22.3	Fig latex	E	-	Food
Lipoxygenase	1.13.11.12	Soybeans	I	-	Food
Papain	3.4.22.2	Pawpaw latex	E	++	Meat
Bacterial enzymes					
a-Amylase	3.2.1.1	*Bacillus*	E	+++	Starch
b-Amylase	3.2.1.2	*Bacillus*	E	+	Starch
Asparaginase	3.5.1.1	*Escherichia coli*	I	-	Health
Glucose isomerase[h]	5.3.1.5	*Bacillus*	I	++	Fructose syrup
Penicillin amidase	3.5.1.11	*Bacillus*	I	-	Pharmaceutical
Protease[i]	3.4.21.14	*Bacillus*	E	+++	Detergent
Pullulanase[j]	3.2.1.41	*Klebsiella*	E	-	Starch

Fungal enzymes					
a-Amylase	3.2.1.1	*Aspergillus*	E	++	Baking
Aminoacylase	3.5.1.14	*Aspergillus*	I	-	Pharmaceutical
Glucoamylase[k]	3.2.1.3	*Aspergillus*	E	+++	Starch
Catalase	1.11.1.6	*Aspergillus*	I	-	Food
Cellulase	3.2.1.4	*Trichoderma*	E	-	Waste
Dextranase	3.2.1.11	*Penicillium*	E	-	Food
Glucose oxidase	1.1.3.4	*Aspergillus*	I	-	Food
Lactase[l]	3.2.1.23	*Aspergillus*	E	-	Dairy
Lipase[e]	3.1.1.3	*Rhizopus*	E	-	Food
Rennet[m]	3.4.23.6	*Mucormiehei*	E	++	Cheese
Pectinase[n]	3.2.1.15	*Aspergillus*	E	++	Drinks
Pectin lyase	4.2.2.10	*Aspergillus*	E	-	Drinks
Protease[m]	3.4.23.6	*Aspergillus*	E	+	Baking
Raffinase[o]	3.2.1.22	*Mortierella*	I	-	Food
Yeast enzymes					
Invertase[p]	3.2.1.26	*Saccharomyces*	I/E	-	Confectionery
Lactase[l]	3.2.1.23	*Kluyveromyces*	I/E	-	Dairy
Lipase[e]	3.1.1.3	*Candida*	E	-	Food
Raffinase[o]	3.2.1.22	*Saccharomyces*	I	-	Food

[a] The names in common usage are given. As most industrial enzymes consist of mixtures of enzymes, these names may vary from the recommended names of their principal component. Where appropriate, the recommended names of this principal component is given below.
[b] The EC number of the principal component.
[c] I - intracellular enzyme; E - extracellular enzyme.
[d] +++ > 100 ton year^{-1}; ++ > 10 ton year^{-1}; + > 1 ton year^{-1}; - < 1 ton year^{-1}.
[e] triacylglycerol lipase;
[f] chymosin;
[a] Endo-1,3(4)-b-glucanase;
[h] xylose isomerase;
[i] subtilisin;
[j] a-dextrin endo-1,6-a-glucosidase;
[k] glucan 1,4-a-glucosidase;
[l] b-galactosidase;
[m] microbial aspartic proteinase;
[n] polygalacturonase;

[o] a-galactosidase;
[p] b-fructofuranosidase.

Lecture 5:

Catalytic properties of enzymes: Introduction

1.4. Catalytic properties of enzymes:

Over-view of Enzymes catalysts

All reaction in the body are mediated by enzymes, which are protein catalysis that increase the rate of reaction without being changed in overall process. Among the many biologic reaction that are energetic possible, Enzyme selectively channel reactant called substrate into useful pathways. Enzymes thus direct all metabolic events.

Enzyme are Protein Catalyst that increase the velocity of the chemical reaction, and are not consumed during the reaction they catalyse. Some type of RNA act like a Enzyme, RNA with catalytic activity are called Ribozymes. Enzymes are protein catalysts**,** they influence the kinetics but not the thermodynamics of a reactionIncrease the rate of a chemical reaction

Properties of enzymes

Enzyme molecules contain a special pocket called a active site. The active site contain amino acid side chain that create a three dimension surface complementary to the substrate .the active site bind the substrate , forming an enzyme substrate (ES) complex. The ES is converted to enzyme product(EP), which subsequently dissociated to enzyme and product.

Catalytic efficiency: Most enzyme catalytic reactionare highly efficient, proceeding from 103 to 108 times faster than the uncatalysed reaction. Eacg enzyme molecule is capable of transforming 100 to 1000 substrate molecules into product each sec. The number of molecules of substrate converted to product is called the turnover no.

Characteristic of Enzymes

Certain substance is small amount have unique capacity of speeding-up chemical reaction without being alter after the reaction, they acceleration the velocity of the reactionwithout necessary initially it. Substances that behave in this manner are called catalyst or catalytic agent. For eg hydrogen and oxygen do not combine to any appreciable extent under normal atmospheric condition. However unlike platinum, while is inorganic, enzyme are organic compound produce by living organism. Thus we may define enzyme as organic catalyst produced by a organic cell.

The three distinctive characteristic are 1)specificity. 2)High Catalyst rate 3)high capacity for regulation.

Dynamic mathematical model in biotechnology require beside the information require the stoichiometry ok the biological reaction system.. The identification of a priori unknown reaction kinetics is often a critical task due to the non-linearity and (over-) parameterization of the model equations introduced to account for all the possible modulation phenomena. The contribution of this paper is to propose a general formulation of reaction kinetics, as an extension of the Michaelis-Menten kinetics, which allows limitation/activation and inhibition effects to be described with a reduced number of parameters.

Enzyme Catalyst

Most of thereactionthat occur in living organism are catalyst by molecule called enzyme. Most enzymes are proteins (certain RNA molecules also act as enzyme).

An enzyme is in specific in its action. Many enzymes catalyst only the conversion of a particular reactant to a particular product;other enzyme catalyst only a certain class of reaction (by ester hydrolysis) . Enzyme speed up reaction rate very substantially and in their absent most biological reactionoccur . The molecule an enzyme act on is called the substrate. The substrate binds to a specific active site on the enzyme, so form as enzyme substrate complex. Some physiological poison act by binding to active site of an enzyme,there blocking the action of the enzyme. The structure of an inhibitor may resemble the structure of enzyme substrate .Cyanide act by blocking the enzyme cytochrome oxidase.

The single called Escherichia coli, a bacterium that flourished in human colons, contain about 2500 different enzymes.

Lecture 6:

Enzyme Kinetics: Michaelis-Menten Kinetics

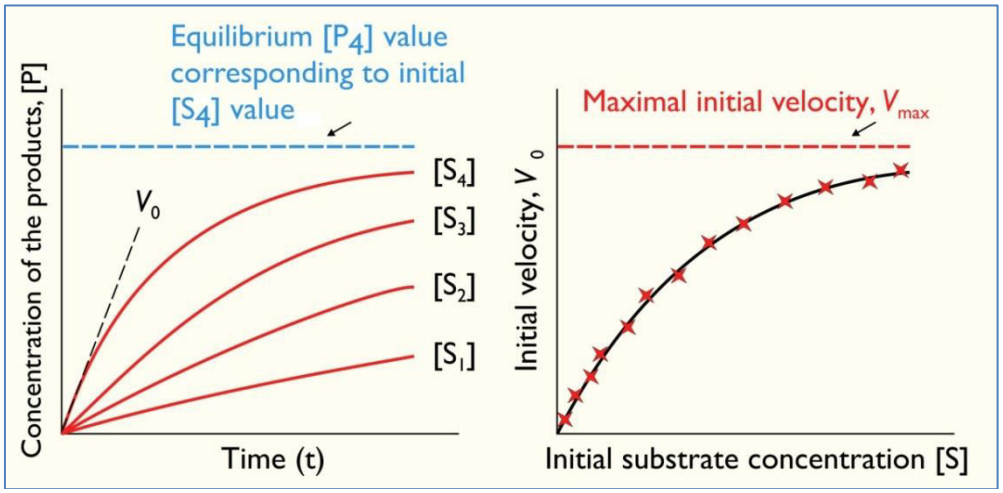
1.5.1.: Michaelis-MentenKinetics:

After the thermodynamic interpretation, we will now review the fundamental relationships of enzyme kinetics. As we focus on enzymatic reactions, the reactant will be denoted as substrate and will be referred to as “S”. The product will be denoted as P as previously. For simplicity, instead of the previous $A + B \rightarrow P$ scheme, we will introduce the simplest $S \rightarrow P$ case.

When the topic of enzyme kinetics first emerged, almost nothing was known about the physical nature of enzymes and the possible mechanisms of rate enhancement.

Let us start with a thought experiment considering the dependence of the rate of a non-catalysed chemical reaction as a function of reactant concentration. In the case of the simplest first-order reaction, the rate of the non-catalysed S→P reaction can be written as V = d[P]/dt = k[S]. In other words, the rate of the reaction is linearly proportional to the concentration of the reactant S. In principle, the rate could be increased to "infinity"—the only limit would be set by the solubility of S.

In typical cases, when an enzyme catalyses the same reaction, the rate is enhanced by orders of magnitude. However, very importantly, the plot of the [S]-V function would be principally different in this latter case. At a constant enzyme concentration, [E], and in the range of low [S] values, increasing [S] would result in an almost linear increase of the rate, V. However, as [S] is increased even further, V would not increase to the same extent and it would ultimately approximate a maximal value limit (Figure 9.3).



Initial rates (V_0) in enzyme kinetic experiments

The first kinetic model that successfully explained this phenomenon was introduced by Leonor Michaelis and Maud Menten. Their presumption, which nowadays might seem trivial, was revolutionary in their time. They assumed that the enzyme directly interacts with the substrate in a stoichiometric manner, the interaction results in a well-defined intermediate complex, and the interaction leads to thermodynamic equilibrium. This scheme is illustrated by Equation 1.1 in which ES denotes the complex. As a tribute to this first successful model, ES has been named the Michaelis complex.

$$E + S \overset{K_S}{\leftrightarrows} ES \overset{k_{cat}}{\rightarrow} E + P \qquad (1.1)$$

The above simplest scheme is based on the following assumptions. The interaction between the substrate and the enzymes that generates the ES complex leads to a (quasi-)equilibrium; and the

reaction is instantaneous, i.e. so fast that the rate constants corresponding to this step do not restrict the overall rate of the reaction. Accordingly, for this first reaction step, the simple model introduces only an equilibrium constant, K_S, and it is not concerned with the two rate constants that determine K_S. K_S—which, in the case of non-covalent E-S interaction, is dissociation constant—is defined by Equation 1.2:

$$K_S = \frac{[E][S]}{[ES]} \qquad (1.2)$$

According to this model, the catalytic rate constant, k_{cat} that corresponds to the rate of the decomposition of ES towards the product, is much lower than the (non-defined) rate constants corresponding to K_S. Accordingly, the rate of ES decomposition towards the product is so low that (at least in the time frame of the measurement) it does not affect the quasi-equilibrium concentrations of [E], [S] and [ES].

Let us see how the initial rate of the reaction depends on substrate concentration if the starting assumptions apply.

The rate equation corresponding to the scheme introduced in Equation 9.18 is shown in Equation 1.3. This equation refers to a first-order reaction in which the rate of the reaction is proportional to the concentration of only a single entity, in this case the ES complex:

$$V_0 = k_{cat}[ES] \qquad (1.3)$$

The scheme does not consider the opposite reaction, i.e. the one in which the interaction of the enzyme and the product would regenerate the ES complex. This is because the model focuses on the very beginning of the reaction when the concentration of the product is negligible. It is therefore of utmost importance that the rates defined in this model are always initial rates that correspond to the (theoretical) zero time point of the reaction.

The concentration of ES is not pre-set by the experimenter, but it can be determined experimentally once the right model is established. In the following steps, we will transform Equation 1.3 into a derived one that contains pre-set enzyme and substrate concentration parameters. To do so, we need to express ES concentration as a function of the pre-set enzyme and substrate concentrations. Let us start with Equation 1.2 that defined the K_S dissociation constant. Then let us consider a self-explanatory relationship shown in Equation 1.4, between the total enzyme concentration, ($[E]_T$), the free enzyme concentration and ES concentration:

$$[E] = [E]_T - [ES] \qquad (1.4)$$

By combining Equation 1.4 with Equation 1.2, we get equation 1.5:

$$K_S = \frac{[E][S]}{[ES]} = \frac{([E]_T - [ES])[S]}{[ES]} = \frac{[E]_T[S] - [ES][S]}{[ES]} \quad (1.5)$$

Multiplying both sides by ES concentration yields equation 1.6:

$$K_S[ES] = [E]_T[S] - [ES][S] \quad (1.6)$$

In Equation 9.24, the ES-containing terms are rearranged to be side by side:

$$K_S[ES] + [ES][S] = [E]_T[S] \quad (1.7)$$

Then, in Equation 9.25, [ES] is multiplied out from the sum of the products:

$$[ES](K_S + [S]) = [E]_T[S] \quad (1.8)$$

Finally, both sides are divided by the multiplying factor of [ES], which results in Equation 1.9:

$$[ES] = \frac{[E]_T[S]}{(K_S + [S])} \quad (1.9)$$

By these algebraic transformations, ES concentration has been expressed as a function of the experimentally pre-set enzyme and substrate concentrations and that of the equilibrium (dissociation) constant.

If the starting conditions are set such that the total substrate concentration exceeds the total enzyme concentration by orders of magnitude, the amount of substrate getting into the ES complex will be negligible compared to the total amount of substrate. Consequently, the free substrate concentration (at the beginning of the reaction) will practically equal the total substrate concentration. This way, both the total enzyme concentration, $[E]_T$, and the free substrate concentration, [S], will be experimentally-set known parameters.

In the next step, based on Equation 1.3, both sides of Equation 1.9 are multiplied by the k_{cat} rate constant to yield the initial rate, according to Equation 1.10:

$$V_0 = k_{cat}[ES] = \frac{k_{cat}[E]_T[S]}{(K_S + [S])} \quad (1.10)$$

Note that the highest achievable initial reaction rate, denoted as V_{max}, will be achieved when all enzyme molecules are incorporated into the ES complex. In this case, the substrate saturates the enzyme molecules. Then, and only then, $[ES] = [E]_T$. According to this, the $k_{cat}[E]_T$ product in

Equation 1.10 will be in fact the value of V_{max}. By taking this into account, we can formulate Equation 1.11, which is the final equation of the simplest enzyme kinetic model:

$$V_0 = \frac{V_{\max}[S]}{(K_S + [S])} \qquad (1.11)$$

This equation is a so-called rectangular hyperbola function that has the following general description: $Y=P_1X/(P_2+X)$, where X is the independent variable, in our case the substrate concentration, Y is the dependent variable, in our case the initial reaction rate, while P_1 and P_2 are the two parameters of the function, in our case the V_{max} and the K_S, respectively. P_1 is also the horizontal asymptote of the hyperbola, the maximal value of Y that the graph of the function approaches as X tends to infinity. The P_2 parameter is K_S.

Note that this equation is in a perfect accordance with the experimental observations regarding the [S]-V_0 relationship illustrated in Figure 9.3: when $[S] << K_S$, [S] becomes negligible in the denominator, leading to Equation 1.12:

$$V_0 = \frac{V_{\max}}{K_S}[S] \qquad (1.12)$$

In Equation 1.12, the multiplying factor of the substrate concentration is the quotient of two constants and, as such, it is also a constant. Accordingly, in the [S] range where the substrate concentration is orders of magnitude lower than the value of K_s, the initial reaction rate will be linearly proportional to substrate concentration, exactly as the experiments show. In other words, in this substrate concentration range the reaction is a (pseudo)-first order reaction in respect of the substrate.

In the other extreme case when $[S] >> K_S$, it is K_S that will be negligible compared to the value of [S] in the denominator of Equation 1.12. Accordingly, if we consider only this [S] range, we get equation 1.13:

$$V_0 = \frac{V_{\max}[S]}{[S]} = V_{\max} \qquad (1.13)$$

In the substrate concentration region where [S] exceeds K_S by orders of magnitude, the substrate saturates the enzyme, i.e. all enzyme molecules will be in the ES complex, and the initial reaction rate reaches a maximal value, i.e. it cannot be further increased by increasing the concentration of the substrate. Accordingly, in this substrate concentration range, the initial reaction rate is practically independent of the substrate concentration, (i.e. it is zero-order in respect of the substrate). When the substrate concentration equals the value of K_S, the initial reaction rate is the half of the V_{max} value.

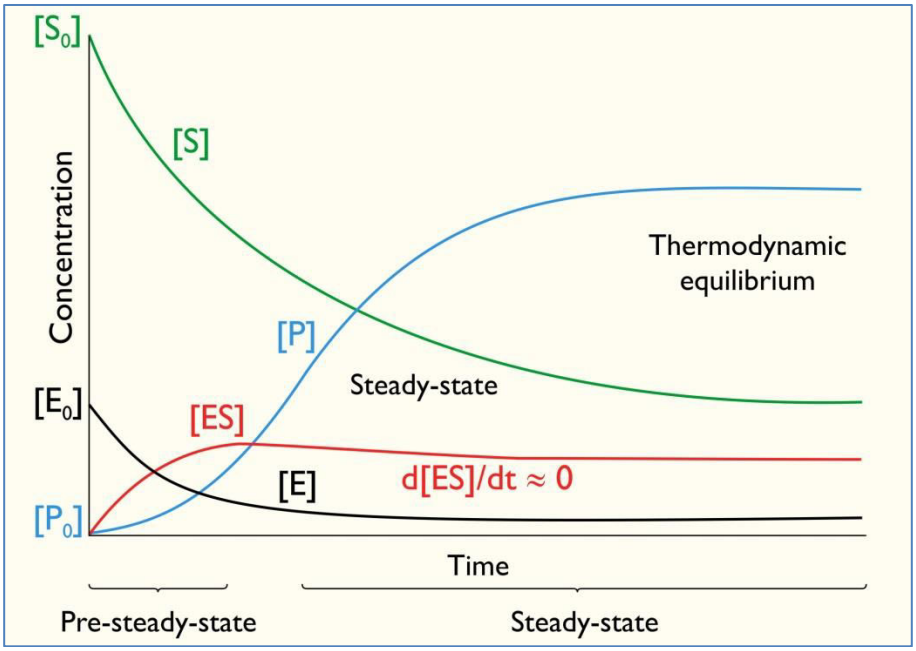
In a simple descriptive way, the deduced Equation 1.11 appears to be in accordance with the observations. Yet, it leads to serious theoretical contradictions. The more efficient the enzyme, the less rational the initial assumptions of the above model. If the enzyme is highly efficient, the rate of ES conversion into product should be very high. If so, that process should interfere with the presumed quasi-equilibrium between the enzyme, the substrate and the ES complex. Therefore, the assumption of equilibrium for the first step of the reaction renders the model ill-suited for describing the action of genuinely efficient enzymes.

The second—and equally significant—problem is that this first model also contradicts the thermodynamic bases of catalysis. K_S is dissociation constant and, as such, it defines the affinity, i.e. the strength of the binding interaction. In the first model, K_S describes how strongly the enzyme binds the substrate or, in other words, how stable the ES complex is. The lower the K_S, the more stable the complex. Moreover, based on the model, the lower the K_S, the more effective the enzyme. This is because a low K_S means that the enzyme reaches half-maximal reaction rate at low substrate concentration. But there is a discrepancy here. By increasing the stability of the interaction between the enzyme and the substrate, the reaction rate should decrease because the enzyme would stabilise the substrate in the ground state. Naturally, the enzyme must bind the substrate, but it should not bind it too tightly. Instead, the enzyme should bind tightly the transition state, thereby decreasing the activation free enthalpy of the reaction.

Due to these contradictions, the first kinetic model had to be developed further. The improved model accounts for the two additional kinetic rate constants that were left out from the first model. One of these, denoted as k_1, corresponds to the formation of the ES complex from free enzyme and substrate. The other, denoted as k_{-1}, corresponds to the reverse reaction, the dissociation of the ES complex towards enzyme and substrate. The improved scheme is illustrated in Equation 1.14:

$$E + S \underset{k_1}{\overset{k_{-1}}{\rightleftarrows}} ES \xrightarrow{k_2} E + P \qquad (1.14)$$

According to the improved model, when the solutions of the enzyme and the substrate are mixed, instead of a dynamic equilibrium, a steady-state will develop very quickly, almost instantaneously. Depending on the exact initial conditions, the steady-state may last long, i.e. the concentration of the ES complex can remain practically constant for a long period of time (exactly, d[ES]/dt is not zero, but much smaller than either d[P]/dt or d[S]/dt). The steady-state requires equal rates for ES generation and ES decomposition. The reaction is triggered by adding the substrate. (Proportions of the figure are not realistic, as otherwise various parts of the figure could not be shown on the same page. In reality, the steady-state can be reached in milliseconds and it can last for minutes. Moreover, in reality, [S] starts from a much higher level because [S] >> [E].)



Decomposition of the ES complex can happen on two different routes: it can occur towards product formation with rate constant k_2, and also in the opposite direction towards substrate regeneration with rate constant k_{-1}. The development of the steady-state is illustrated in above Figure while the mathematical requirements of steady-state formation are formulated by Equation 1.15:

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_2[ES] = 0 \qquad (1.15)$$

Although the steady-state modification of the model was suggested by George Edward Briggs and John Burdon Sanderson Haldane, the improved model is still being referred to as the kinetic model of Michaelis and Menten and the derived equation as the Michaelis-Menten equation. In this equation, the ES complex forms at a rate k_1[E][S]; it decays back towards the substrate at a rate k_{-1}[ES] and decomposes towards the product at a rate k_2[ES]. In the steady-state, the rate of ES formation and the sum of the two types of ES decomposition rates are equal in magnitude and, thus, the concentration of the ES complex does not significantly change.

In the next section, Equation 1.15 will be transformed in several steps in order to yield a final equation containing the experimentally pre-set enzyme concentration and substrate concentration. The improved model has the same initial requirement: the substrate concentration must exceed the enzyme concentration by orders of magnitude. The rate of the reaction can be easily formulated, as shown in Equation 1.16, which is analogous to Equation 1.3 of the simpler model:

$$V_0 = k_2[ES] \qquad (1.16)$$

As the upgraded model contains three rate constants instead of one, the earlier model's k_{cat} is replaced with the k_2 rate constant introduced in the improved scheme. By rearranging Equation 1.15 we get Equation 1.17:

$$k_1[E][S] = k_{-1}[ES] + k_2[ES] \tag{1.17}$$

Just as it was done in the first model, the free enzyme concentration is expressed as the difference of the total enzyme concentration and the concentration of the ES complex (Equation 1.17):

$$k_1([E]_T - [ES])[S] = k_{-1}[ES] + k_2[ES] \tag{1.17}$$

Simple algebraic transformations result in Equation 1.18:

$$k_1[E]_T[S] - k_1[ES][S] = (k_{-1} + k_2)[ES] \tag{1.18}$$

Then the [ES] term-containing parts are organised to the same side, resulting in Equation 1.19:

$$k_1[E]_T[S] = (k_{-1} + k_2)[ES] + k_1[ES][S] \tag{1.19}$$

By multiplying out the [ES] factor, we get equation 1.20:

$$k_1[E]_T[S] = (k_{-1} + k_2 + k_1[S])[ES] \tag{1.20}$$

In Equation 1.21, the [ES] term is arranged to the left side:

$$[ES] = \frac{k_1[E]_T[S]}{k_{-1} + k_2 + k_1[S]} \tag{1.21}$$

Then, on the right side of the equation, both the numerator and the denominator are divided by the k_1 rate constant, which results in Equation 1.22:

$$[ES] = \frac{[E]_T[S]}{\frac{(k_{-1} + k_2)}{k_1} + [S]} \tag{1.22}$$

In the denominator of Equation 1.22, there is a complex composed of three rate constants. This quotient has been defined as the Michaelis constant, with the abbreviation K_M. Replacing the quotient for $K_M$yields Equation 1.23:

$$\frac{(k_{-1}+k_2)}{k_1} \equiv K_M \qquad (1.23)$$

Note that K_M is defined as the ratio of the two-direction decay rate of the ES complex and the one-direction formation rate of the complex. In other words, it quantifies the instability of the ES complex.

Building Equation 1.21 into Equation 1.22 leads to Equation 1.24:

$$[ES] = \frac{[E]_T[S]}{K_M + [S]} \qquad (1.24)$$

By combining Equations 1.16 and 1.24, we get Equation 1.25:

$$V_0 = k_2[ES] = \frac{k_2[E]_T[S]}{(K_M + [S])} \qquad (1.25)$$

As already mentioned, in the case of Equation 1.9 of the simpler model, the highest initial rate (at a given total enzyme concentration), V_{max}, is achieved when all enzyme molecules are part of an ES complex, i.e. $[ES] = [E_T]$. Therefore, the $k_2[E]_T$ product in Equation 1.25 is in fact the V_{max}. Taking this into consideration, we get Equation 1.26, which is the final and most commonly used rate equation of the improved Michaelis-Menten model:

$$V_0 = \frac{V_{\max}[S]}{(K_M + [S])} \qquad (1.26)$$

Note that the mathematical forms of the corresponding final equations of the simple (Equation 1.11) and the improved (Equation 1.26) models are identical. Consequently, just like Equation 1.11, Equation 1.26 is also consistent with experimental results in terms of the dependence of the initial rate on substrate concentration. In the substrate concentration range where $[S] << K_M$, V_0 is linearly proportional to [S]; while in the range where $[S] >> K_M$, the reaction rate does not depend on the substrate concentration, and it has a maximal value.

When the numerical value of [S] equals that of K_M, the rate of the reaction is exactly half of the maximal one.

In spite of the many formal similarities, there are principal differences in the interpretations of the two models. Note that the K_S constant of the simple model and the K_M constant of the improved model have different meanings. Equation 1.27 illustrates how the dissociation constant-type K_S is derived from the k_1 and k_{-1} rate constants.

$$K_S = \frac{k_{-1}}{k_1} \qquad (1.27)$$

As already mentioned, K_S is a kind of affinity descriptor indicating how tightly the enzyme binds the substrate. It is readily apparent from the comparison of Equations 1.27and 1.23 that K_M will equal $K_S$only if the $k_2 << k_{-1}$ requirement is fulfilled. This requirement would mean that the rate of ES decomposing towards the product would be orders of magnitude lower than the rate of ES decomposing back to substrate. However, for efficient enzymes, the very opposite situation, i.e. $k_2 >> k_{-1}$, may apply. As already mentioned, low values of K_S would—by mathematical formalism—indicate an efficient enzyme. However, from a thermodynamic aspect, the very same characteristics would indicate an inefficient enzyme.

The more efficient the enzyme, the less correct it is to interpret K_M as indicating affinity of the enzyme-substrate interaction.

Clearly, K_M and K_S of the two models are not interchangeable. On the other hand, the k_2 rate constant of the improved model, if the ES complex decays to enzyme and product in a single step, is identical to the k_{cat} rate constant of the simpler model. (If the final product is formed in a complex multi-step process, the k_{cat} rate constant is derived from an equation containing the rate constants of all individual steps.)

Let us examine the meaning of the k_{cat} rate constant based on Equations 1.28 and 1.29:

$$V_{\max} = \left(\frac{d[P]}{dt}\right)_{\max} = k_{cat}[E]_T \qquad (1.28)$$

$$k_{cat} = \frac{\left(\frac{d[P]}{dt}\right)_{\max}}{[E]_T} \qquad (1.29)$$

As it was shown, the maximal rate is the product of k_{cat} and the total enzyme concentration. Equation 1.29 is obtained when the rate of product concentration change is divided by the total enzyme concentration. As the product and the enzyme are present in the very same solution having the same volume, the ratio of concentrations is identical to the ratio of the numbers of molecules. Therefore, Equation 1.29 will provide a value on the number of product molecules generated by a single enzyme molecule in a unit period of time.

Accordingly, the k_{cat} rate constant is also referred to as the turnover number of the enzyme. It has the dimension of reciprocal of time. Naturally, the higher the value of k_{cat}, the more efficient the enzyme as a “chemical” catalyst, working after the ES complex had already been formed. Let us also note that, in cases when in the improved model $k_{cat} = k_2$, this rate constant is present in the

nominator of the quotient defining K_M. This means that a high k_{cat} will increase the value of K_M. On the other hand, it is also clear that a lower K_M means that the half-maximal reaction rate is achieved at a lower substrate concentration, which is another measure of being an efficient enzyme. What would then be the best parameter to describe enzyme efficiency?

The most effective enzymes are expected to catalyse the reaction at a high rate even at low substrate concentration and, naturally, their turnover number should also be high. As already explained, at low substrate concentration where [S] << K_M, the reaction rate is a linear function of substrate concentration. This is shown in Equation 1.30:

$$V_0 \cong \frac{k_{cat}}{K_M}[E][S] \qquad (1.30)$$

The k_{cat}/K_M quotient in the equation is the rate constant of a second-order reaction, as the rate of this reaction depends on the concentration of two compounds. This k_{cat}/K_M quotient illustrates how efficiently the enzyme performs in the most challenging situation when the substrate concentration is very low. Accordingly, the k_{cat}/K_M quotient can be called *catalytic efficiency*, although its commonly used denomination is *specificity constant*.

Let us examine what sets the limit of the catalytic efficiency of enzymes by reviewing the case of the most efficient enzymes. Starting with Equation 1.30, we can generate Equation 9.49 by replacing the k_{cat}term with k_2 according to the simple one-step scheme of the reaction, and by replacing K_M (by definition) with the $(k_{-1}+k_2)/k_1$ quotient:

$$V_0 = \frac{k_2}{\frac{(k_{-1}+k_2)}{k_1}}[E][S] = \frac{k_2 k_1}{k_{-1}+k_2}[E][S] \approx k_1[E][S] \qquad (1.31)$$

In the right side of the resulting equation, and in the special case when $k_2 >> k_{-1}$, the k_{-1} term becomes negligible in the denominator. By ignoring the k_{-1} term, both the numerator and the denominator of the resulting quotient can be divided by the k_2 term. The $k_2 >> k_{-1}$ situation means that the ES complex decays practically exclusively towards the product instead of returning towards the substrate. When this assumption is valid, the k_{cat}/K_M rate constant of the reaction approximates the value of k_1. This means that the limit for the rate of the enzymatic reaction will be set by the rate at which the enzyme and the substrate encounter. Naturally, the steady-state rate of the reaction cannot exceed the rate of ES formation.

At first approximation, the limit for the most effective enzymes is set by the diffusion that limits the speed of the enzyme-substrate encounters. The diffusion rate can be accurately calculated based on the size of the diffusing molecules and the viscosity of the medium. In the case of some

extremely efficient enzymes, it turned out that the rate of the enzymatic reaction exceeded the calculated diffusion rate.

There are at least two situations that can explain this apparent discrepancy. If the substrate carries large number of electric charges of one type and the substrate binding site of the enzyme carries complementary (opposite) electrical charges, then the electrostatic attraction can steer the substrate towards the enzyme. The electrostatic interaction between full charges is a long-range interaction (compared to the size of small molecules). This condition, together with the orienting capacity of the interaction, significantly increases the frequency of enzyme-substrate encounters relative to the simple diffusion-limited case. A second important exception from the simple diffusion limit is related to multi-enzyme complexes. Many “final products” of metabolic or other chemical pathways are formed by a series of consecutive chemical reactions that proceed through many intermediate compounds. This means that the product of a given enzymatic reaction is the substrate of another enzyme catalysing the subsequent reaction. In a simple case, the product of the first reaction must encounter the second enzyme by random diffusion. If, on the other hand, these enzymes are organised in a proper arrangement in multi-enzyme complexes, the intermediate compounds can be “handed” directly from one enzyme to the other. This channelling effect can greatly enhance the overall rates of multi-step pathways.

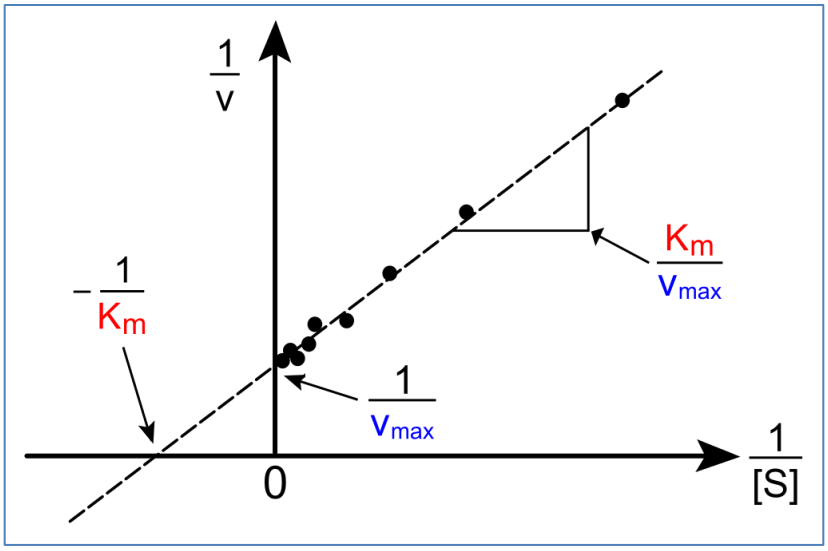
(ref:http://elte.prompt.hu/sites/default/files/tananyagok/IntroductionToPracticalBiochemistry/ch09s02.html)

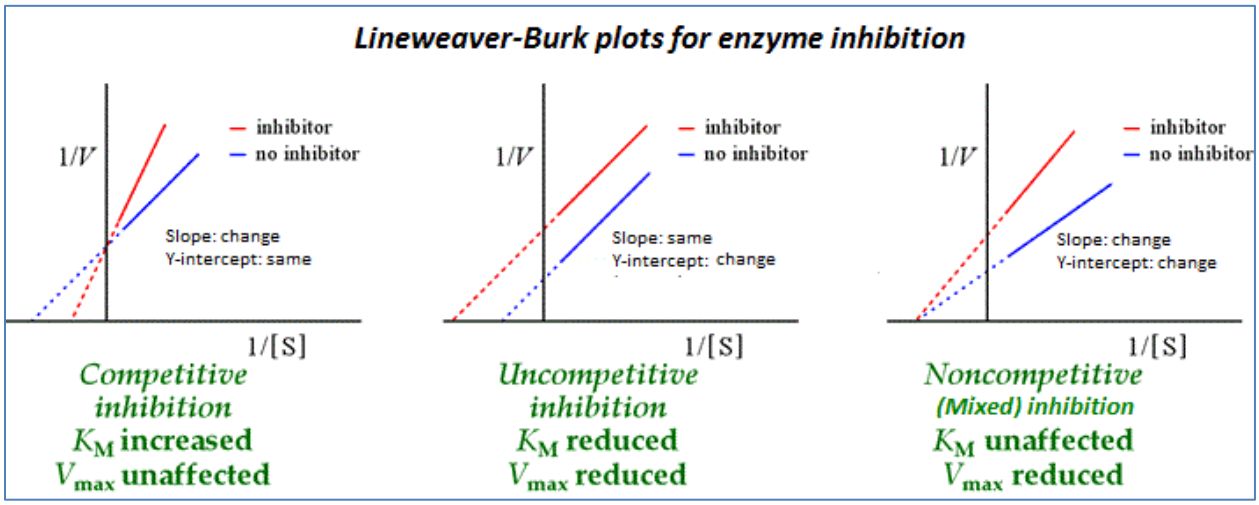
Lecture 7

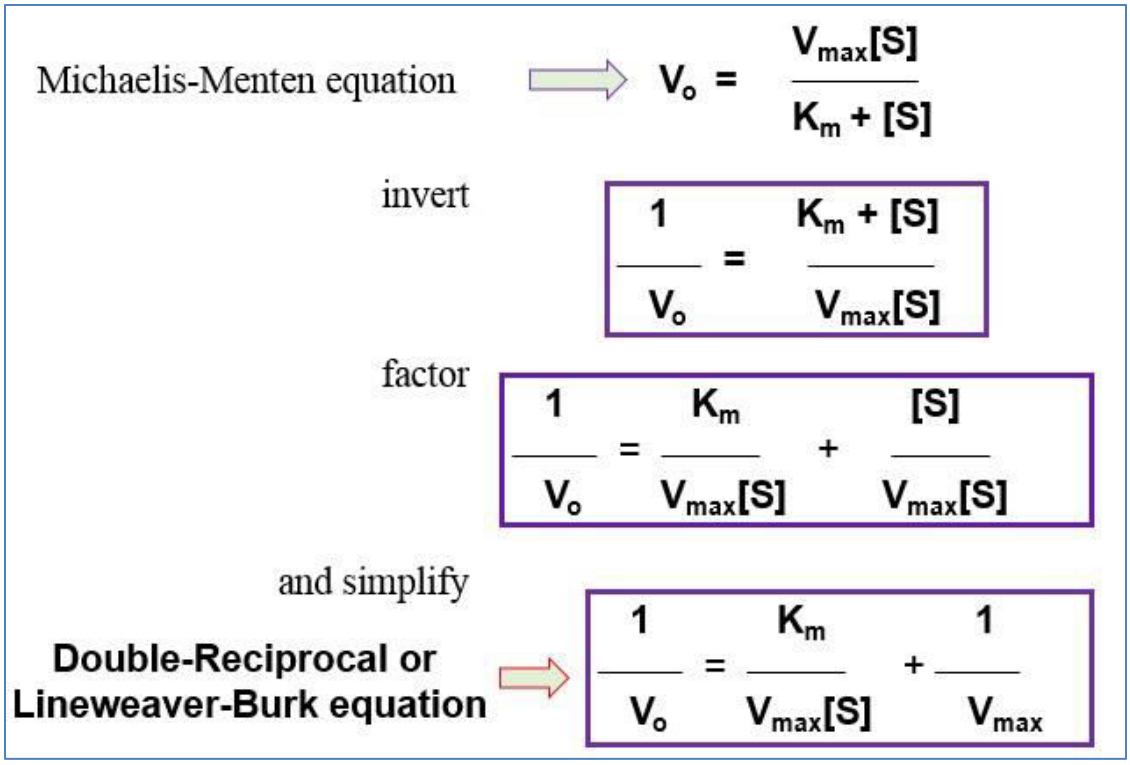
Enzyme Kinetics: Lineweaver- Burk Equation, Effect of Temperature and pH on Enzyme Activity

1.5.2. Lineweaver- Burk Equation

In biochemistry, the **Lineweaver–Burk plot** (or **double reciprocal plot**) is a graphical representation of the Lineweaver–Burk equation of enzyme kinetics, described by Hans Lineweaver and Dean Burk in 1934.


1/v
-1/Km
Km/Vmax
1/Vmax
0
1/[S]

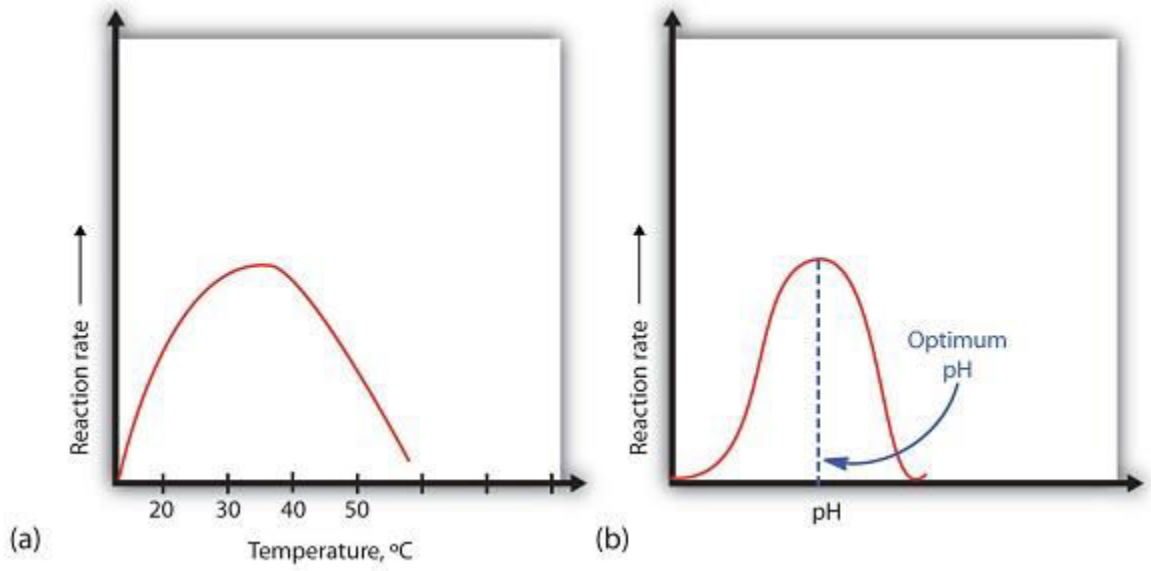

Lineweaver-Burk plots for enzyme inhibition
1/V
inhibitor
no inhibitor
Slope: change
Y-intercept: same
1/[S]
Competitive inhibition
KM increased
Vmax unaffected
1/V
inhibitor
no inhibitor
Slope: same
Y-intercept: change
1/[S]
Uncompetitive inhibition
KM reduced
Vmax reduced
1/V
inhibitor
no inhibitor
Slope: change
Y-intercept: change
1/[S]
Noncompetitive
(Mixed) inhibition
KM unaffected
Vmax reduced



1.5.3. Effect of Temperature and pH on enzyme activity:

1.5.3.1. Effect of Temperature:

A general rule of thumb for most chemical reactions is that a temperature rise of 10°C approximately doubles the reaction rate. To some extent, this rule holds for all enzymatic reactions. After a certain point, however, an increase in temperature causes a decrease in the reaction rate, due to denaturation of the protein structure and disruption of the active site (part (a) of Figure . For many proteins, denaturation occurs between 45°C and 55°C. Furthermore, even though an enzyme may appear to have a maximum reaction rate between 40°C and 50°C, most biochemical reactions are carried out at lower temperatures because enzymes are not stable at these higher temperatures and will denature after a few minutes.



Temperature and pH versus Concentration. (a) This graph depicts the effect of temperature on the rate of a reaction that is catalyzed by a fixed amount of enzyme. (b) This graph depicts the effect of pH on the rate of a reaction that is catalyzed by a fixed amount of enzyme.

At 0°C and 100°C, the rate of enzyme-catalyzed reactions is nearly zero. This fact has several practical applications. We sterilize objects by placing them in boiling water, which denatures the enzymes of any bacteria that may be in or on them. We preserve our food by refrigerating or freezing it, which slows enzyme activity. When animals go into hibernation in winter, their body temperature drops, decreasing the rates of their metabolic processes to levels that can be maintained by the amount of energy stored in the fat reserves in the animals' tissues.

1.5.3.2. Effect of Hydrogen Ion Concentration (pH):

Because most enzymes are proteins, they are sensitive to changes in the hydrogen ion concentration or pH. Enzymes may be denatured by extreme levels of hydrogen ions (whether high or low); *any* change in pH, even a small one, alters the degree of ionization of an enzyme's acidic and basic side groups and the substrate components as well. Ionizable side groups located in the active site must have a certain charge for the enzyme to bind its substrate. Neutralization of even one of these charges alters an enzyme's catalytic activity.

An enzyme exhibits maximum activity over the narrow pH range in which a molecule exists in its properly charged form. The median value of this pH range is called the optimum pH of the enzyme (part (b) of Figure). With the notable exception of gastric juice (the fluids secreted in the stomach), most body fluids have pH values between 6 and 8. Not surprisingly, most enzymes exhibit optimal activity in this pH range. However, a few enzymes have optimum pH values outside this range. For example, the optimum pH for pepsin, an enzyme that is active in the stomach, is 2.0.

Lecture 8

Intracellular and extra-cellular enzymes

1.6. Intracellular and extra-cellular enzymes:

Intracellular enzyme:
An endoenzyme, or intracellular enzyme, is an enzyme that functions within the cell in which it was produced. Majority of enzymes fall within this category. It is possible for a single enzyme to have both endoenzymatic and exoenzymatic functions. Enzymes that act inside cells are responsible for catalysing the millions of reactions that occur in metabolic pathways such as **glycolysis** in the mitochondria and in the**photosynthetic pathway** in the chloroplast. The lysosome contains many enzymes that are mainly responsible for destroying old cells.
Example: Glycolytic enzymes, enzymes of Kreb's Cycle.

Extracellular enzyme:
An exoenzyme, or extracellular enzyme, is an enzyme that is secreted by a cell and functions outside of that cell. Exoenzymes are produced by both prokaryotic and eukaryotic cells and have been shown to be a crucial component of many biological processes. Most often these enzymes are involved in the breakdown of larger macromolecules. Example: amylase, pectinase, lipase etc.

The enzymes that function in our digestive systems are manufactured in cells - but work extracellularly. Spiders and flies are two examples of animals that have taken extracellular digestion a step further. They secrete an enzyme soup into or on their food. In spiders, this is injected into the prey's body. The enzyme soup digests the prey's body contents (specific enzymes breaking down proteins to amino acids, lipids into fatty acids and glycerol and polysaccharides into monosaccharides) and the spider simply sucks up the resulting already digested food. Saprophytic fungi also secrete enzymes through their hyphal tips in order to digest their food.

Module II 8L

Lecture 9

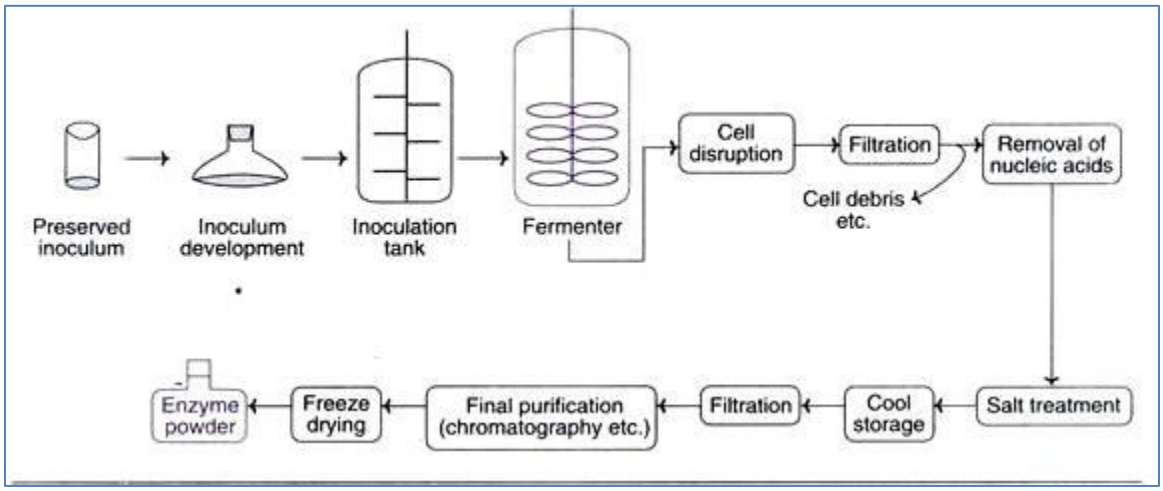
The Technology of Enzyme Production—General Considerations

2.1. The Technology of Enzyme Production—General Considerations:

In general, the techniques employed for microbial productions of enzymes are comparable to the methods used for manufacture of other industrial products .The salient features are briefly described.

1. Selection of organisms
2. Formulation of medium
3. Production process
4. Recovery and purification of enzymes.

An outline of the flow chart for enzyme production by microorganisms is depicted in Fig.



Steps of Industrial Enzyme Production

Selection of organism:

The most important criteria for selecting the microorganism are that the organism should produce the maximum quantities of desired enzyme in a short time while the amounts of other metabolite produced are minimal. Once the organism is selected, strain improvement for optimising the enzyme production can be done by appropriate methods (mutagens, UV rays). From the organism chosen, inoculum can be prepared in a liquid medium.

Formulation of medium:

The culture medium chosen should contain all the nutrients to support adequate growth of microorganisms that will ultimately result in good quantities of enzyme production. The ingredients of the medium should be readily available at low cost and are nutritionally safe. Some of the commonly used substrates for the medium are starch hydrolysate, molasses, corn steep liquor, yeast extract, whey, and soy bean meal. Some cereals (wheat) and pulses (peanut) have also been used. The pH of the medium should be kept optimal for good microbial growth and enzyme production.

Production process:

Industrial production of enzymes is mostly carried out by submerged liquid conditions and to a lesser extent by solid-substrate fermentation. In submerged culture technique, the yields are more and the chances of infection are less. Hence, this is a preferred method. However, solid substrate fermentation is historically important and still in use for the production of fungal enzymes e.g. amylases, cellulases, proteases and pectinases.

The medium can be sterilized by employing batch or continuous sterilization techniques. The fermentation is started by inoculating the medium. The growth conditions (pH, temperature, O_2 supply, nutrient addition) are maintained at optimal levels. The froth formation can be minimised by adding antifoam agents.

The production of enzymes is mostly carried out by batch fermentation and to a lesser extent by continuous process. The bioreactor system must be maintained sterile throughout the fermentation process. The duration of fermentation is variable around 2-7 days, in most production processes. Besides the desired enzyme(s), several other metabolites are also produced. The enzyme(s) have to be recovered and purified.

Recovery and purification of enzymes:

The desired enzyme produced may be excreted into the culture medium (extracellular enzymes) or may be present within the cells (intracellular enzymes). Depending on the requirement, the commercial enzyme may be crude or highly purified. Further, it may be in the solid or liquid form. The steps involved in downstream processing i.e. recovery and purification steps employed will depend on the nature of the enzyme and the degree of purity desired.

In general, recovery of an extracellular enzyme which is present in the broth is relatively simpler compared to an intracellular enzyme. For the release of intracellular enzymes, special techniques are needed for cell disruption. The reader must invariably refer them now and learn all the details, as they form part of enzyme technology. Microbial cells can be broken down by physical means (sonication, high pressure, glass beads). The cell walls of bacteria can be lysed by the enzyme lysozyme. For yeasts, the enzyme β-glucanase is used. However, enzymatic methods are expensive.

The recovery and purification (briefly described below) steps will be the same for both intracellular and extracellular enzymes, once the cells are disrupted and intracellular enzymes are released. The most important consideration is to minimise the loss of desired enzyme activity.

Removal of cell debris:
Filtration or centrifugation can be used to remove cell debris.

Removal of nucleic acids:
Nucleic acids interfere with the recovery and purification of enzymes. They can be precipitated and removed by adding poly-cations such as polyamines, streptomycin and polyethyleneimine.

Enzyme precipitation:
Enzymes can be precipitated by using salts (ammonium sulfate) organic solvents (isopropanol, ethanol, and acetone). Precipitation is advantageous since the precipitated enzyme can be dissolved in a minimal volume to concentrate the enzyme.

Liquid-liquid partition:
Further concentration of desired enzymes can be achieved by liquid-liquid extraction using polyethylene glycol or polyamines.

Separation by chromatography:
There are several chromatographic techniques for separation and purification of enzymes. These include ion-exchange, size exclusion, affinity, hydrophobic interaction and dye ligand chromatography .Among these, ion- exchange chromatography is the most commonly used for enzyme purification.

Drying and packing:
The concentrated form of the enzyme can be obtained by drying. This can be done by film evaporators or freeze dryers (lyophilizers). The dried enzyme can be packed and marketed. For certain enzymes, stability can be achieved by keeping them in ammonium sulfate suspensions.

All the enzymes used in foods or medical treatments must be of high grade purity, and must meet the required specifications by the regulatory bodies. These enzymes should be totally free from toxic materials, harmful microorganisms and should not cause allergic reactions.

Lecture 10

Regulation of Microbial Enzyme Production —General Considerations

2.2. Regulation of Microbial Enzyme Production —General Considerations:

A maximal production of microbial enzymes can be achieved by optimising the fermentation conditions (nutrients, pH, O_2, temperature etc.). For this purpose, a clear understanding of the genetic regulation of enzyme synthesis is required. Some of the general aspects of microbial enzyme regulation are briefly described.

Induction:

Several enzymes are inducible i.e. they are synthesized only in the presence of inducers. The inducer may be the substrate (sucrose, starch, galactosides) or product or intermediate (fatty acid, phenyl acetate, xylobiose). A selected list of inducible enzymes and the respective inducers is given in Table .

Enzyme	*Inducer*
Invertase	Sucrose
Amylase	Starch
Lipase	Fatty acids
β-Galactosidase	Galactosides
Penicillin G amidase	Phenylacetate
Xylanase	Xylobiose

The inducer compounds are expensive and their handling (sterilization, addition at specific time) also is quite difficult. In recent years, attempts are being made to develop mutants of microorganisms in which inducer dependence is eliminated.

Feedback repression:

Feedback regulation by the end product (usually a small molecule) significantly influences the enzyme synthesis. This occurs when the end product accumulates in large-quantities. Large scale production of feedback regulated enzymes is rather difficult. However, mutants that lack feedback repression have been developed to overcome this problem.

Nutrient repression:

The native metabolism of microorganism is so devised that there occurs no production of unnecessary enzymes. In other words, the microorganisms do not synthesize enzymes that are not required by them, since this is a wasteful exercise. The inhibition of unwanted enzyme production is done by nutrient repression. The nutrients may be carbon, nitrogen, phosphate or sulfate suppliers in the growth medium. For large scale production of enzymes, nutrient repression must be overcome.

Glucose repression is a classical example of nutrient (more appropriately catabolite) repression. That is in the presence of glucose, the enzymes needed for the metabolism of rest of the compounds are not synthesized. Glucose repression can be overcome by feeding of carbohydrate to the fermentation medium in such a way that the concentration of glucose is almost zero at any given time. In recent years, attempts are being made to select mutants that are resistant to catabolite repression by glucose. For certain microorganisms, other carbon sources such as pyruvate, lactate, citrate and succinate also act as catabolite repressors.

Nitrogen source repression is also observed in microorganisms. This may be due to ammonium ions or amino acids. Most commonly inexpensive ammonium salts are used as nitrogen sources. The repression by ammonium salts can be overcome by developing mutants resistant to this nitrogen source.

(Ref: http://www.biologydiscussion.com/enzymes/enzyme-technology/enzyme-technology-application-and-commercial-production-of-enzymes/10185)

Lecture 11

Variables Affecting the Oxygen Supply Rate during Fermentation

2.3. Variables Affecting the Oxygen Supply Rate during Fermentation:

Delivering oxygen to cells:

In cell culture, oxygen is a key substrate for growth, production, and maintenance activities. Cells obtain their oxygen in free and noncompound forms, called dissolved oxygen (DO). One of the most important functions of bioreactors is providing dissolved oxygen to cells continuously through a process called aeration.
Aeration in the bioreactor typically occurs when:

a. Oxygen diffuses through overlay to the cell culture medium interface.

b. Oxygen from the spargers dissolves in the cell culture through convection with the help of agitation.

Agitation disperses the oxygen bubbles and promotes mass transfer of the gas bubbles through the gas-liquid (cell culture medium) interface. The rate of oxygen transfer (OTR) from gas to liquid interface is a function of physicochemical properties of the cell culture medium, the geometrical parameters of the bioreactor, and presence of cells.

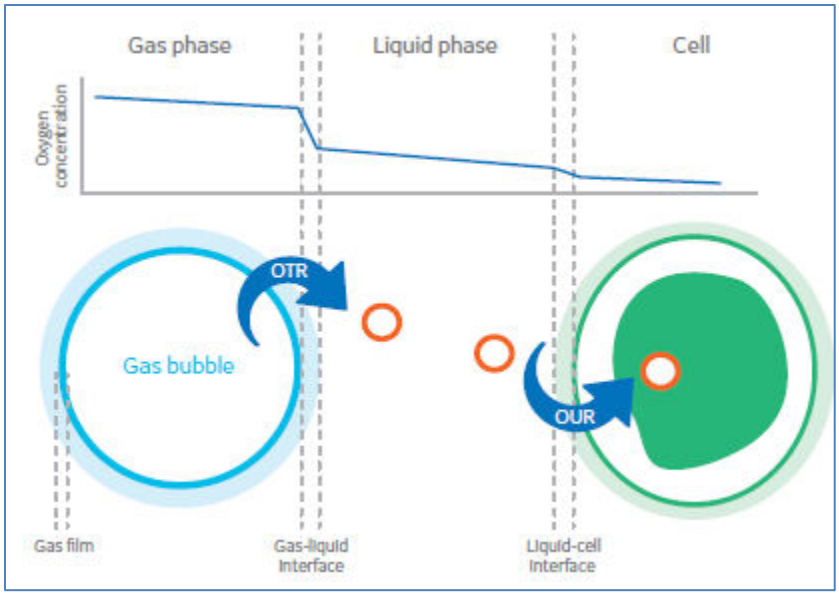


Diagram of a gas bubble in liquid, showing how the bubble is released, solubilized, and transferred to a cell

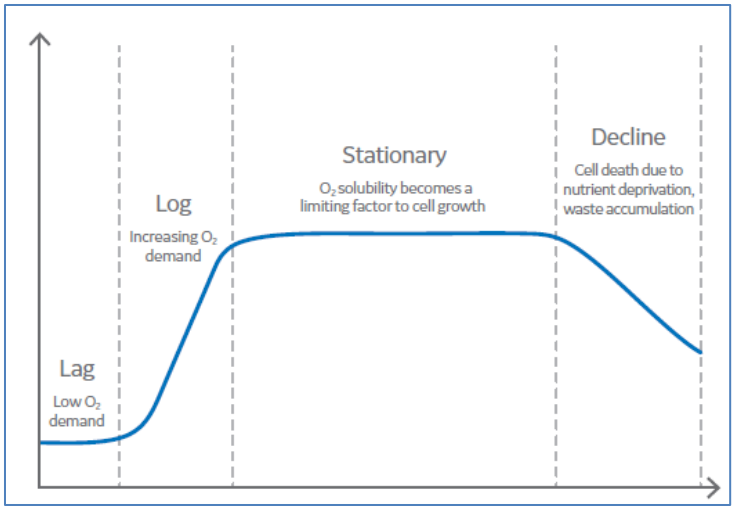
Oxygen utilization rate (OUR) is often cell line-dependent. The following table lists the rates for common industrial cell lines (Ruffieux, P. A. et al. and Xiu, Z. L. et al.)

Oxygen utilization rates of cell lines typically used in biomanufacturing

Cell line	**OUR [10^{-13}mol/cell/h]**
DG44 (CHO)	2
CHO	5.0–8.04
NSO (myeloma)	2.19–4.06
MAK (hybridoma)	4.16
FS-4 (human diploid cells)	0.5
HFN7.1 (hybridoma)	2

Due to its low solubility in liquid phase and increasing metabolic consumption by the cells with time, oxygen is supplied continuously to the cell culture. Oxygen supply is carefully controlled for optimal cell growth by manipulating bioreactor parameters.

During batch cell culture, OUR (or OTR) is initially low during the lag phase, where cells are self-synthesizing and there is little gain of cell density. As cell density increases during the exponential phase, OUR increases until OTR becomes a limiting rate, as determined by the mass transfer of oxygen into the bulk liquid.



Phases of cell growth

The OTR and OUR rates are correlated by the oxygen mass transfer coefficient, kLa. Therefore, the OTR, through its correlation to kLa, defines a theoretical maximum cell density that could be achieved in cell culture.

Utility of kLa values

Because of this association with cell density, kLa values are particularly useful in the following scenarios:

Scenario 1: Evaluating scalability within the same bioreactor platform

The conventional scale-up of bioprocesses is based on physicochemical and geometric similarity. kLa is kept constant for this scenario. The OTR should remain constant for a bioreactor platform with geometric similarity (such as Xcellerex bioreactors). Bioreactor physical characteristics at the different scales are altered to provide the necessary OTR at controlled temperature, pH, and DO to achieve the target cell density.

Scenario 2: Technical transfer across different bioreactor designs

During the comparison, kLa is utilized as a target performance metric when a process is transferred from one bioreactor platform to another design. Bioreactor hardware design (e.g., stirrer geometry and aeration-sparger option) and running parameters (e.g., gas flow rate or power input) are altered to achieve a similar kLa, providing a similar cell density.

Equation for kLa

Imagine a gas bubble in liquid. For this discussion the gas bubble contains oxygen, and the liquid is the liquid in a bioreactor. kLa can be represented by the following equation:

$$kLa = kL \times a$$

- Where kLa is the mass transfer coefficient from the gas to liquid phase, given in sec^{-1}
- kL = liquid side mass transfer coefficient (resistance in gas side film can be neglected)
- a = bubble surface (available for diffusion)

Key variables that impact kLa values

Any change to process and engineering parameters or to physical characteristics will have an impact on kLa and should be considered when evaluating bioreactor platforms and performing scaling calculations.

Here are four key variables that can affect kLa values:

i . Gas bubble size

When gas bubble size decreases, surface area and gas residency time increases, causing bubbles to stay in the culture longer. Thus, there is a greater opportunity for oxygen to release mass transfer into the cell culture medium. An increase in this oxygen residence time improves kLa.

ii. Mixing

In a bioreactor, mixing is used to eliminate gradients of concentration (cell, gas, medium, and nutrient), temperature, and other properties. Mixing time is widely used to characterize mixing efficiency in a bioreactor. Mixing efficiency is one of the most significant factors affecting both performance and scale-up in a bioreactor.

Gas bubble size and residency time are highly dependent upon three mixing conditions: impeller type, speed, and location(s). kLa values generally increase as tip speed increases. However, tip speed is proportional to shear forces that can lead to cell death. Bioreactors, therefore, are designed with different impeller types, combinations, and locations to achieve target kLa values without creating these shear forces.

Generally, kLa values are closely associated with impeller design, with Rushton typically higher than paddle, which is typically higher than marine and pitched impeller.

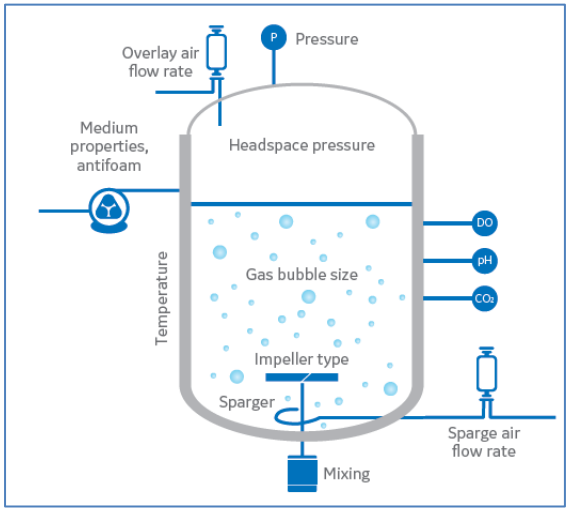


Diagram of a bioreactor process showing key factors that can influence kLa values

iii. Air flow rate

Higher oxygen availability drives kLa increases. Increasing oxygen supply to a bioreactor drives this availability and can be controlled by modifying concentration (air vs O_2 enrichment) and

volumetric flow. Although high kLa values are desirable, it is important to consider the actual operating conditions and implications to cell viability and associated process costs.

For example, high air flow rates can cause cell damage due to shear forces. Excessive foam might also be generated, requiring a high concentration of antifoam that could hinder downstream processing. Additionally, higher air flow rates require a larger exhaust filter area, driving consumable cost increases.

iv. Properties of the liquid or medium

During cell culture, small bubbles collide and coalesce to form larger bubbles, decreasing surface area (a) and subsequently kLa. Be aware of reported kLa values in which high salt concentrations are used, because this can prevent bubble coalescing. Antifoaming agents are used to influence surface tension, resulting in reduced bubble coalescence and foaming.

However, this principle does not always lead to increases in OTR wherein antifoam also reduces bubble mobility, which subsequently reduces the kLa (Doran, P.).

Other factors that affect cell culture kLa

v. Measurement method

Several different methods are used. Most commonly the nitrogen stripping (i.e., gassing-out) method is employed.

When scaling a process within the same platform, it is important to use an identical method for measuring kLa. kLa, when combined with process engineering parameters (i.e., tip speed, power input), can be used to experimentally determine the cell density in a larger bioreactor compared with a smaller bioreactor.

vi. Temperature

Increasing temperatures inversely affects both the volumetric mass transfer coefficient and oxygen solubility in culture medium. Oxygen solubility in pure water falls with increasing temperature (i.e., -0.5×10^{-3} kg/m^{-3} between 35°C and 30°C; Doran, P.).

Therefore, it is important to note the temperature conditions from vendor-supplied characterization data.

vii. Sparger characteristics

kLa values will vary widely with sparger characteristics, including number, pore size, and surface area, because these factors affect bubble size, gas velocity, and flow rates.

(Ref: https://www.gelifesciences.com/en/us/solutions/bioprocessing/knowledge-center/7-factors-that-affect-oxygen-transfer-to-cells-in-bioreactors)

Lecture 12

Scale-Up and Scale-Down of a Fermenter

2.4. Scale-Up and Scale-Down of a Fermenter:

Scale-up means increasing the scale of fermentation, for example from the laboratory scale to the pilot plant scale or from the pilot plant scale to the production scale. Increase in scale means an increase in volume and the problems of process scale-up are due to the different ways in which process parameters are affected by the size of the unit. It is the task of the fermentation technologist to increase the scale of fermentation without a decrease in yield or, if a yield reduction occurs, to identify the factor which gives rise to the decrease and to rectify it. The major factors involved in scale-up are:

(i) Inoculum development: An increase in scale may mean that extra stages have to be incorporated into the inoculum development programme. This aspect is considered in Chapter 6.
(ii) Sterilization: Sterilization is a scale dependent factor because the number of contaminating micro-organisms in a fermenter must be reduced to the same absolute number regardless of scale. Thus, when the scale of a process is increased the sterilization regime must be adjusted accordingly, which may result in a change in the quality of the medium after sterilization.

(iii) Environmental parameters: The increase in scale may result in a changed environment for the organism. These environmental parameters may be summarized as follows:

(a) nutrient availability,

(c) temperature,

(d) dissolved oxygen concentration,

(e) shear conditions,

(f) dissolved carbon dioxide concentration,

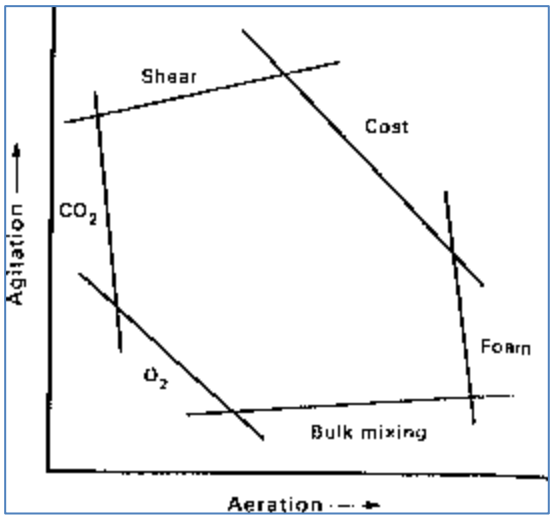
(g) foam production.

All the above parameters are affected by agitation and aeration, either in terms of bulk mixing or the provision of oxygen. Points a, b, c and e are related to bulk mixing whilst d, e, f and g are related to air flow and oxygen transfer. Thus, agitation and aeration tends to dominate the scale-up literature. However, it should always be remembered that inoculum development and sterilization difficulties may be the reason for a decrease in yield when a process is scaled up and that achieving the correct aeration/agitation regime is not the only problem to be addressed.

Scale-up of aeration/agitation regimes in stirred tank reactors

From the list of environmental parameters affected by aeration and agitation it will be appreciated that it is extremely unlikely that the conditions of the small-scale fermentation will be replicated precisely on the large scale. Thus, the most important criteria for a particular

fermentation must be established and the scale-up based on reproducing those characteristics. The two axes of Fig. are agitation and aeration and the zone within the hexagon represents suitable aeration/agitation regimes. The boundary of the hexagon is defined by the limits of oxygen supply, carbon dioxide accumulation, shear damage to the cells, cost, foam formation and bulk mixing. For example, the agitation rate must fall between a minimum and maximum value — mixing is inadequate below the minimum level and shear damage to the cells is too great above the maximum value. The limits for aeration are determined at the minimum end by oxygen limitation and carbon dioxide accumulation and at the maximum end by foam formation. The shape of the window will depend on the fermentation — for exam-



The 'scale-up' window defining the operating boundaries for aeration and agitation in the scale-up of a fermentation. After Fox (1978) reproduced from Lilly (1983).

The solution of the scale-up problem is three-fold:

(i) The identification of the principal environmental domain affected by aeration and agitation in the fermentation, e.g. oxygen concentration, shear, bulk mixing.

(ii) The identification of a process variable (or variables) which affects the identified environmental domain.

(iii) The calculation of the value of the process variable to be used on the large scale which will result in the replication of the same environmental conditions on both scales.

Hubbard (1987) and Hubbard et al. (1988) summarized the procedure for scaling up both Newtonian and non-Newtonian fermentations and proposed two methods to determine the large scale conditions:

Method 1

(i) Determine the volumetric air flow rate (Q) on the large scale based on maintaining Q/V constant (V = working volume of the fermenter).

(ii) Calculate the agitator speed that will give the same KLa on the large scale; this is achieved using the correlations between power consumption and N and between Kta and power consumption.

Method 2

(i) Calculate the agitator speed keeping the impeller tip speed constant, vrND,.

(ii) Calculate Q from power correlations and K, a correlations.

The accuracy of these scale-up techniques is only as good as the power and Kha correlations, so it is worth expending some considerable time to test the validity of potential correlations for the fermentation in question.

(Ref: https://www.buffalobrewingstl.com/micro-organisms/scaleup-and-scaledown.html)

Lecture 13

Industrial Amylase Production: Overview

2.5.1. Industrial Amylase Production: Overview:

What are Amylases?

- Enzymes that break down starch or glycogen.
- Present in the saliva of humans and some other mammals, where it begins the chemical process of digestion.
- All amylases are glycoside hydrolases and act on α-1,4-glycosidic bonds.
- Produced by a variety of living organisms, ranging from bacteria to plants and humans.

Classification Of Amylases is based on how they break down starch molecules:

1. α-Amylase
2. β-Amylase
3. γ-Amylase

Advantages and Uses of Microbial Amylase:

- production of amylases is in economical bulk production capacity.
- microbes are also easy to manipulate to obtain enzymes of desired characteristics.
- The microbial amylases meet industrial demands a large number of them are available commercially.
- useful applications in food, brewing, textile, detergents and pharmaceutical industries.
- In detergents production, they are applied to improve cleaning effect and are also used for starch de-sizing in textile industry.

- mainly employed for starch liquefaction (a process of dispersion of insoluble starch in aqueous solution) to reduce their viscosity, production of maltose, oligosaccharide mixtures.

Alpha-amylase producing bacteria :

- *Bacillus subtilis*
- *B. amyl liquefacient*
- *B. polymyxin*
- *B. coagulans*
- *B. licheniformis*
- *Micrococcus, Lactobacillus*

Alpha- amylase producing fungi:

- *Aspergillus*
- *Penicillium*
- *Cephalosporium*
- *Mucor*
- *Cladosporium*
- *Candida*

Among them commercially used strains are:

- *Bacillus subtilis*
- *B. licheniformis*
- *B. amyloliquefaciens*
- *A. Oryza*

Lecture 14

Production Procedure and Industrial Application of Alpha- Amylase

2.5.2. Production Procedure of Alpha- Amylase:

Production procedure of bacterial alpha-amylase:

Bacterial alpha-amylase is produced by either solid state fermentation or by submerged state fermentation. The process of production of enzyme may be discussed under the following headings;

i. **Producing organism:** Amylase is produced by bacteria ,fungi, plants, and animals but due to the advantages of microorganism in the production process microorganisms have substantial potential to contribute different and many industrial applications, Due to the rapid growth rate of Bacillus species it is more predominant in the production of amylase enzyme industrially.
   The most commonly used commercial strains are *Bacillus substilis*, *Bacillus licheniformis*, *B. amyloliquefaciens*. These strains are improved by mutation to yield enzyme of about 250 times greater than that of wild strains.

ii. **Media:** Mostly complex wheat bran, a rice bran , gram husk, mustard oilseed cake which are known as agro-industrial wastes are used in the production of amylase in the case of solid state fermentation but production by submerged state method requires media

optimization and includes 5% starch, ammonium nitrate, sodium citrate, $MgSO_4.7H_2SO_4$., $CaCO_3.2H_2O$, peptone, Yeast extract etc are used.

iii. **Inoculum preparation:** Inoculum is prepared by shake flask culture method . The inoculum is then grown in small fermenters before final fermentation. Thus selected microbial strains are ready for inoculation and inoculated in nutrient broth and then followed by the incubation for 24 hours at temperature 37° C to obtain standard inoculum.

iv. **Fermenters:** The fermenter used in the submerged state fermentation process may betray or stirred type.

v. **Fermentation:** Mode of fermentation; It is carried out either in batch or fed-batch manner.

The difference between batch fermentation and fed-batch fermentation:

Batch fermentation	Fed-batch fermentation
1) During the fermentation process if the bioreactor is feed once that means no more feeding after this is known as batch fermentation.	1) In this type of fermentation, the controlled addition of nutrients in the reactor is used at certain time is known as fed-batch fermentation.
2) We cannot control the rate of growth of organisms controlling environmental parameters.	2) We cannot control a rate of growth of organisms as once the reactor is feed then it stops with the completion of fermentation or the reaction.
3) It is less usable in the industries.	3) They are the most popular process in the industries.

- **Size of fermenter**: A 100m3 volume of the tank in submerged state fermentation is used.
- **pH :** The optimum pH for the production of amylase is 6-8
- **Temperature:** Maximal enzyme production occurs at a relatively lower temperature of about 27- 30° C. but it is strain specific eg; Thermophilic such as Thermonospora produces the maximum enzyme when the temperature is 53° C.
- **Aeration** : Aeration is done in range of 0.8-1 vvm
- **Duration:** It should be kept for 1-2 days that means for 48 hours.

The enzyme fermentation rate is very low during the exponential phase of growth but just before the rate of growth decreases and spore formation begins amylase production increases.

Production of fungal amylase:

The production of fungal alpha-amylase is mostly carried out by solid state fermentation using wheat bran as the substrate. However, the substrate may contain 8% starch, sodium nitrate , magnesium sulphate, KCL, $FeSO_4$., malt extract are added to optimize the media.

Production method:

i. **Inoculum:** Fungal spores are produced in solid media to use as inoculum. The major fungus used is *Aspergillusoryzae.*

ii. **Fermenter:** Drum fermenter or tray chamber are used.

iii. **Fermentation:** The inoculum of *Aspergillusoryzae* is inoculated into the fermenter and the temperature is maintained at 28 -30°C. The duration of fermentation is 3-5 days.

iv. **Product recovery**: The fermentation broth is separated into liquid and solid part by means of filtration or centrifugation and is purified in subsequent steps for fungal amylase filtration alone is sufficient to separate solid from liquid while for bacterial amylase filtration and centrifugation are carried out.

2.6. Industrial Application of Alpha- Amylase:

Applications Of Industrial Amylases:

ENZYMES	SOURCE	APPLICATIONS
Alpha- amylase	Bacterial α amylase (e.g., Bacillus subtilis), Fungal α amylase (e.g., Aspergillus niger)	Textiles, starch syrups, laundry and dishwashing detergents, paper desizing , fermentation
ß- amylase	From a strain of Bacillus	Brewing, maltose syrup
γ-Amylase	Aspergillus niger	Manufacture of dextrose syrup and high fructose syrup production.

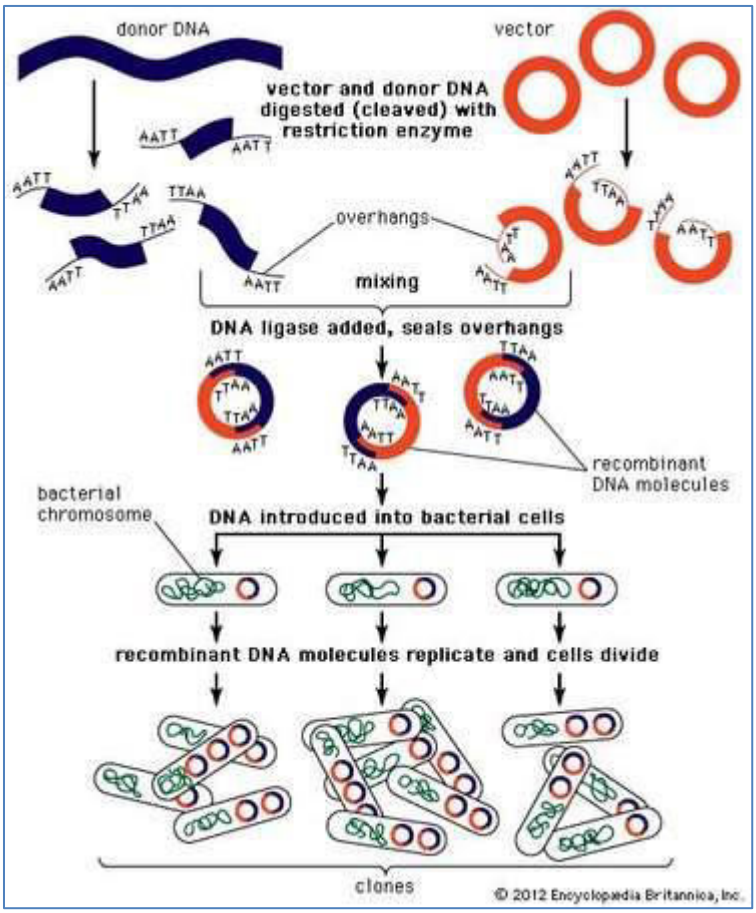
Lecture 15

Recombinant DNA Technology: Definition and Step Involved

2.6.1. Recombinant DNA Technology: Definition:

In biology a clone is a group of individual cells or organisms descended from one progenitor. This means that the members of a clone are genetically identical, because cell replication produces identical daughter cells each time. The use of the word *clone* has been extended to recombinant DNA technology, which has provided scientists with the ability to produce many copies of a single fragment of DNA, such as a gene, creating identical copies that constitute a DNA clone. In practice the procedure is carried out by inserting a DNA fragment into a small DNA molecule and then allowing this molecule to replicate inside a simple living cell such as a bacterium. The small replicating molecule is called a DNA vector (carrier). The most commonly used vectors are plasmids (circular DNA molecules that originated from bacteria), viruses, and yeast cells. Plasmids are not a part of the main cellular genome, but they can carry genes that provide the host cell with useful properties, such as drug resistance, mating ability, and toxin production. They are small enough to be conveniently manipulated experimentally, and, furthermore, they will carry extra DNA that is spliced into them.

2.6.2. Step Involved in Recombinant DNA Technology:



Steps involved in the engineering of a recombinant DNA molecule

Step Involved in Recombinant DNA Technology:

i. Selection and isolation of DNA insert

ii. Selection of suitable cloning vector

iii. Introduction of DNA-insert into vector to form rec DNA molecule

iv. rec DNA molecule is introduced into a suitable host.

v. Selection of transformed host cells.

vi. Expression and multiplication of DNA-insert in the host.

(i) Selection and isolation of DNA insert:

First step in rec DNA technology is the selection of a DNA segment of interest which is to be cloned. This desired DNA segment is then isolated enzymatically. This DNA segment of interest is termed as DNA insert or foreign DNA or target DNA or cloned DNA.

(ii) Selection of suitable cloning vector:

A cloning vector is a self-replicating DNA molecule, into which the DNA insert is to be integrated. A suitable cloning vector is selected in the next step of rec DNA technology. Most commonly used vectors are plasmids and bacteriophages.

(iii) Introduction of DNA-insert into vector to form recDNA molecule:

The target DNA or the DNA insert which has been extracted and cleaved enzymatically by the selective restriction endonuclease enzymes [in step (i)] are now ligated (joined) by the enzyme ligase to vector DNA to form a rec DNA molecule which is often called as cloning-vector-insert DNA construct.

(iv) Recombinant DNA molecule is introduced into a suitable host:

Suitable host cells are selected and the rec DNA molecule so formed [in step (iii)] is introduced into these host cells. This process of entry of rec DNA into the host cell is called transformation. Usually selected hosts are bacterial cells like E. coli, however yeast, fungi may also be utilized.

(v) Selection of transformed host cells:

Transformed cells (or recombinant cells) are those host cells which have taken up the recDNA molecule. In this step the transformed cells are separated from the non-transformed cells by using various methods making use of marker genes.

(vi) Expression and Multiplication of DNA insert in the host:

Finally, it is to be ensured that the foreign DNA inserted into the vector DNA is expressing the desired character in the host cells. Also, the transformed host cells are multiplied to obtain sufficient number of copies. If needed, such genes may also be transferred and expressed into another organism.

Lecture 16

Biological Tools for Recombinant DNA Technology

2.6.3. Important biological tools for recombinant DNA technology are:

(A) Enzymes:i. Restriction Endonucleases

ii. Exonucleases

iii.DNA ligases

iv. DNA polymerase

(B) Cloning Vector

(C) Host organism

(D) DNA insert or foreign DNA

(E) Linker and adaptor sequences

An account of all these biological tools of genetic engineering is given below:

(A) ENZYMES:

A number of specific enzymes are utilized to achieve the objectives of rec DNA technology.

The enzymology of genetic engineering includes the following types of enzymes:

i. Restriction Endonuclease:

These enzymes serve as important tools to cut DNA molecules at specific sites, which is the basic need for rec DNA technology.

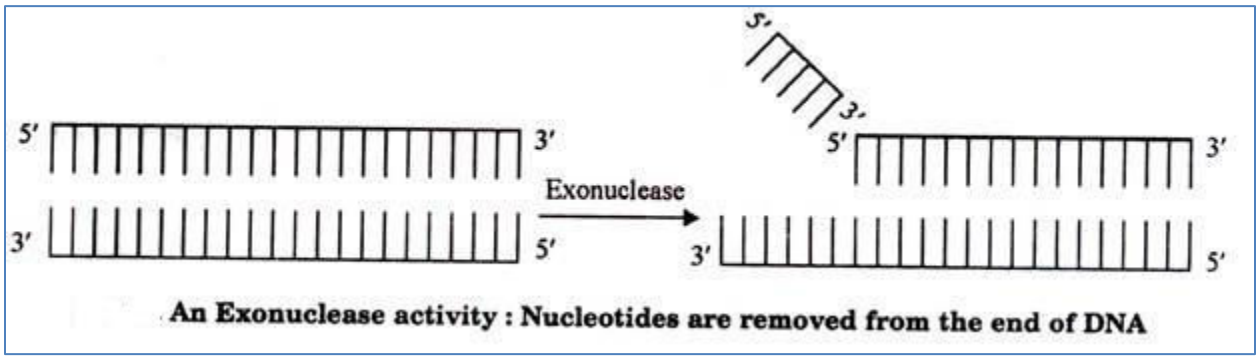
These are the enzymes that produce internal cuts (cleavage) in the strands of DNA, only within or near some specific sites called recognition sites/recognition sequences/ restriction sites 01 target sites. Such recognition sequences are specific for each restriction enzyme. Restriction endonuclease enzymes are the first necessity for rec DNA technology.

The presence of restriction enzymes was first of all reported by W. Arber in the year 1962. He found that when the DNA of a phage was introduced into a host bacterium, it was fragmented into small pieces. This led him to postulate the presence of restriction enzymes. The first true restriction endonuclease was isolated in 1970s from the bacterium *E. coli* by Meselson and Yuan.

Another important breakthrough was the discovery of restriction enzyme Hind-II in 1970s by Kelly, Smith and Nathans. They isolated it from -the bacterium Haemophilus influenza. In the year 1978, the Nobel Prize for Physiology and Medicine was given to Smith, Arber and Nathans for the discovery of endonucleases.

ii. Exonucleases:

Exonuclease is an enzyme that removes nucleotides from the ends of a nucleic acid molecule. An exonuclease removes nucleotide from the 5′ or 3′ end of a DNA molecule. An exonuclease never produces internal cuts in DNA.



An Exonuclease activity : Nucleotides are removed from the end of DNA

In rec DNA technology, various types of exonucleases are employed like Exonuclease Bal31, E. coli exonuclease III, Lambda exonuclease, etc.

Exonculease Bal31 are employed for making the DNA fragment with blunt ends shorter from both its ends.

E coli Exonuclease III is utilized for 3'end modifications because it has the capability to remove nucleotides from 3′-OH end of DNA.

Lambda exonuclease is used to modify 5′ ends of DNA as it removes the nucleotides from 5′ terminus of a linear DNA molecule.

iii. DNA ligase:

The function of these enzymes is to join two fragments of DNA by synthesizing the phosphodiester bond. They function to repair the single stranded nicks in DNA double helix and

in rec DNA technology they are employed for sealing the nicks between adjacent nucleotides. This enzyme is also termed as molecular glue.

iv. DNA polymerases:

These are the enzymes which synthesize a new complementary DNA strand of an existing DNA or RNA template. A few important types of DNA polymerases are used routinely in genetic engineering. One such enzyme is DNA polymerase which, prepared from E coli. The Klenow fragment of DNA polymerase-I .s employed to make the protruding ends double-stranded by extension of the shorter strand.

Another type of DNA polymerase used in genetic engineering is Taq DNA polymerase which is used in PCR (Polymerase Chain Reaction).

Reverse transcriptase is also an important type of DNA polymerase enzyme for genetic engineering. It uses RNA as a template for synthesizing a new DNA strand called as cDNAa e complementary DNA). Its main use is in the formation of cDNA libraries. Apart from all these above mentioned enzymes, a few other enzymes also mark their importance in genetic engineering.

(B) Cloning Vectors:

It is another important natural tool which geneticists use in rec DNA technology. The cloning vector is the DNA molecule capable of replication in a host organism, into which the target DNA is introduced producing the rec DNA molecule.

A cloning vector may also be termed as a cloning vehicle or earner DNA or simply as a vector or a vehicle a great variety of cloning vectors are present for use with E. coli is the host organism.

However under certain circumstances it becomes desirable to use different host for cloning experiments. So, various cloning vectors have been developed based on other bacteria like Bacillus, Pseudomonas, Agrobacterium, etc. and on different eukaryotic organisms like yeast and other fungi.

The cloning vector which has only a single site for cutting by a particular restriction endormclease is considered as a good cloning vector. Different types of DNA molecules may be used as cloning vehicles such as they may be plasmids, bacteriophages, cosmids, phasmids or artificial chromosomes.

(C) Host Organism:

A good host organism is an essential tool tor genetic engineering. Most widely used host for rec DNA technology is the bacterium E. coli. because cloning and isolation of DNA inserts is very easy in this host. A good host organism is the one winch easy to transform and in which the replication of rec DNA is easier. There should not be any interfering element against the replication of rec DNA in the host cells

(D) DNA Insert or Foreign DNA:

The desired DNA segment which is to be cloned is called as DNA insert or foreign DNA or target DNA. The selection of a suitable target DNA is the very first step of rec DNA technology. The target DNA (gene) may be of viral, plant, animal or bacterial origin.

Following points must be kept in mind while selecting the foreign DNA:

- CD It can be easily extracted from source.
- It can be easily introduced into the vector.
- The genes should be beneficial for commercial or research point of view.

A number of foreign genes are being cloned for benefit of human beings. Some of these DNA inserts are the genes responsible for the production of insulin, interferon's, lymphotoxins various growth factors, interleukins, etc.

(E) Linker and Adaptor Sequences:

Linkers and adaptors are the DNA molecules which help in the modifications of cut ends of DNA fragments. These can be joined to the cut ends and hence produce modifications as desired.

Both are short, chemically synthesized, double stranded DNA sequences. Linkers have (within them) one or more restriction endonuclease sites and adaptors have one or both sticky ends. Different types of linkers and adaptors are used for different purposes.

Module III 8L

Lecture 17

Cell disintegration Processes: Introduction and Mechanical Physical Disruption Processes

3.1. Cell disintegration Processes: Introduction:

Cell disruption is an essential part of biotechnology and the downstream processes related to the manufacturing of biological products. The disruption of cells is necessary for the extraction and retrieval of the desired products, as cell disruption significantly enhances the recovery of biological products. Cell disruption cannot be considered an isolated process, as it affects the physical properties of the cell slurry, thus indirectly influencing further downstream processes. Several types of cell disruption methods exist, as biological products may be extracellular, intracellular or periplasmic. Cell disruption methods can be categorised into mechanical methods and non-mechanical methods (Figure 1). Mechanical methods are divided into solid shear methods and liquid shear methods. Non-mechanical methods can be divided into physical methods, chemical methods and enzymatic methods. This report will discuss some methods from each category, as examples of the varying disruption methods available.

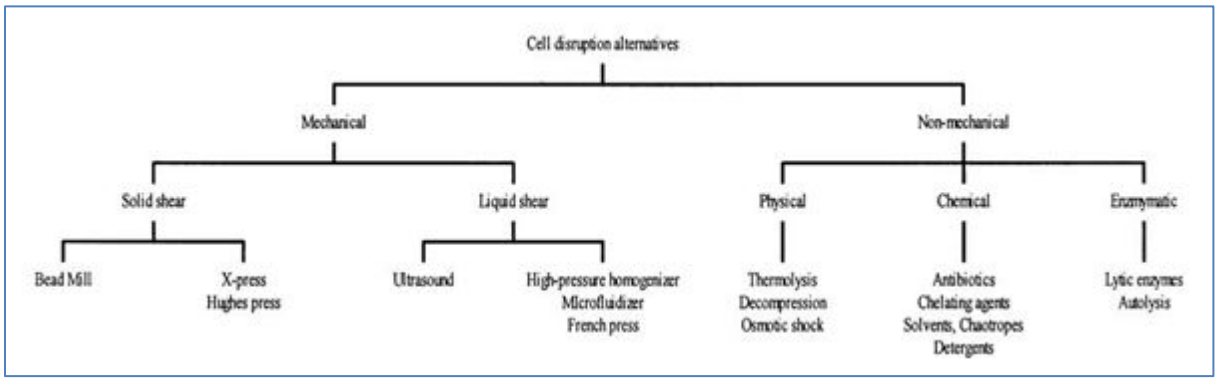


Figure 1. Methods of microbial cell disruption (Geciova J., 2002)

3.2. Mechanical physical methods:

The main principle of the mechanical disruption methods is that the cells are being subjected to high stress via pressure, abrasion with rapid agitation with beads, or ultrasound. (Geciova, 2002) Some methods of disruption are cavitation, shearing, impingement, or combination of those. Intensive cooling of the suspension after the treatment is required in order to remove the heat that was generated by the dissipation of the mechanical energy. Some high-pressure methods can only be applied in laboratory scale, such as French press and Hughes press. For industrial use, the bead mill and high-pressure homogenizer, are suitable. (APV, 2009)

3.2.1. Bead mill:

Bead mills have been originally used in the paint industry, and have been adapted for cell disruption in both small scale and large scale production. (Geciova J., 2002). It is an efficient way of disrupting different microbial cells as different designs have been developed. The main principle requires a jacketed grinding chamber with a rotating shaft, running in its center (figure 2). Agitators are fitted with the shaft, and provide kinetic energy to the small beads that are present in the chamber. That makes the beads collide with each other. The choice of bead size and weight is greatly dependent on the type of cells. The diameter can affect the efficiency of cell disruption in relation of the location of the desired enzyme in the cell. The increased number of beads increases the degree of disruption, due to the increased bead-to-bead interaction. The increased number of beads, however, also affects the heating and power consumption. An optimal condition for bead load is considered between 80 and 85% of the free volume. The discs run at a speed of 1500-2250 rpm. Glass beads with a diameter greater than 0.5 mm are considered best for yeast cells, and diameter lesser than 0.5 mm is optimal for bacterial cells. (APV, 2009) The process variables are: agitator speed, proportion of the beads, beads size, cell suspension concentration, cell suspension flow rate, and agitator disc design. (Chisti Y., 1986).

Main issues related to bead mills, are the high temperature rises with increase of bead volume, poor scale-up, and most importantly, there is a high chance of contamination. (Harrison S., 1991).

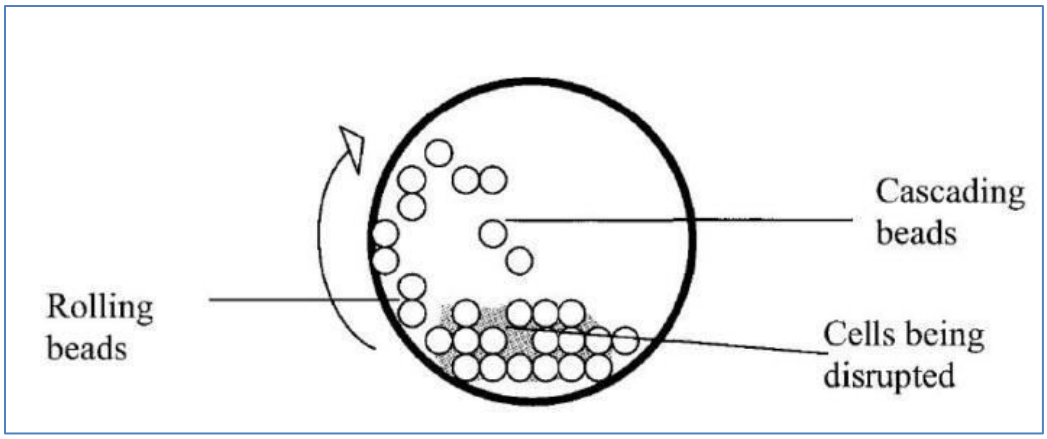


Figure 2: The basic principle of a bead mill

3.2.2. Ultrasound:

Ultrasonic disruption is caused by ultrasonic vibrators that produce a high frequency sound with a wave density of about 20 kHz/s (figure 3). A transducer then converts the waves into mechanical oscillations through a titanium probe, which is immersed into the cell suspension. Such a method is used for both bacterial and fungal cell disruption. Bacterial cell can be disrupted in 30 to 60 sec, and yeast between 2 and 10min. This method is usually used in combination with a chemical method (mostly lysis). (Harrison S., 1991)

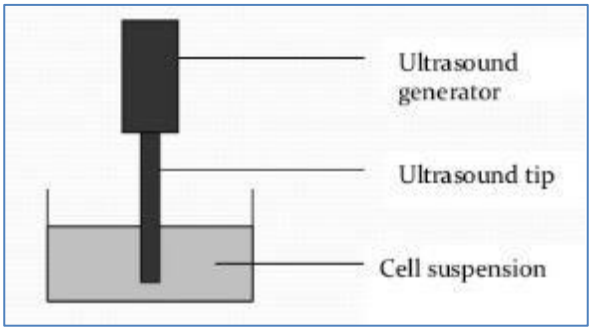


Figure 3: Schematic representation of ultrasonic disruption

Sonication can be very effective in small scale work; however, upscaling is very poor. It has high energy requirements, as well as high health and safety issues, due to noise. It is not continuous. (Chisti Y., 1986)

3.2.3. French press and high pressure homogenizer:

In a French press, or high pressure homogenization, the cell suspension is drawn through a valve into a pump cylinder (figure 4). Then it is forced under pressure of up to 1500 bar, through a narrow annular gap and discharge valve, where the pressure drops to atmospheric. Cell disruption is achieved due to the sudden drop in pressure upon the discharge, causing the cells to explode. This method is one of the most widely known and used methods. It is mostly used for yeast cells. It is a vital unit in the dairy production industry, for milk homogenization. (Middleberg A., 1995) By operating the press at higher pressures, the number of passes of the slurry through it can be decreased in order to obtain the desired degree of disruption.

The French press is a small scale method, whereas the homogenizer can be applied to a large scale production. Homogenisers can vary in design and has a high amount of solids, up to 50% of the feed. Heat generation is also high – 1.5ºC/1000 psi. (Geciova J., 2002)

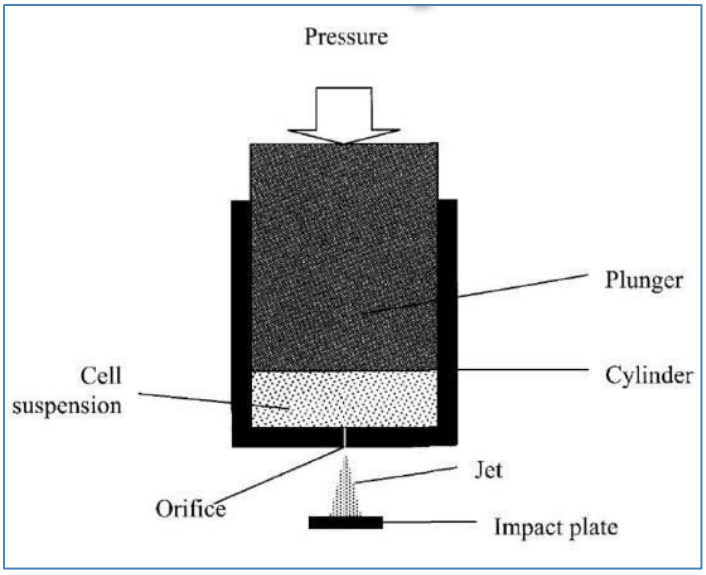


Figure: 4: Schematic representation of the basic principle of a French press

(Ref: CHEM-E3140 Bioprocess Technology II)

Lecture 18

Non-Mechanical Physical Methods of Cell Disruption

3.3. Non-mechanical physical methods:

3.3.1. Thermolysis:

Thermolysis has shown potential in becoming more common in large scale production. Periplasmic proteins in G(-) bacteria are released when the cells are heated up to 50ºC. Cytoplasmic proteins can be released from *E.coli*within 10min at 90 ºC. Improved protein release has been obtained after short high temperature shocks, than when at longer temperature exposures at lower values. Unfortunately, the results are highly unreliable, as the protein solubility changes with temperature fluctuations. (Middleberg A., 1995)

Freezing and thawing of a cell slurry can cause the cells to burst due to the formation and melting of ice crystals. Gradual freezing, leading to the formation of larger crystals, can cause an extensive damage to the cell. By combining this method with cell grinding, this technique has shown great results. However, it is very costly, and restricted to small-scale laboratories. Some reports have also shown loss of enzyme activity. (Harrison S., 1991).

3.3.2. Decompression:

During explosive decompression, the cell suspension is mixed with pressurized subcritical gas for a specified time, depending on the cell type. The gas enters the cell and expends on release, causing the cell to burst. Decompression has been used in small scale laboratories for the disruption of *E.coli.* The technique has shown promising results with yeasts, where it has the advantage that supercritical CO2 is able to extract off-flavours that are caused by lipid components. This technique is proving to be promising, being gentle on the cells, resulting in large debris that are easier to remove in order to obtain the desired product. Downsides, however, include its low efficiency and its high dependency on pressure release and time of contact between the cell suspension and the gas. Decompression chamber is shown in figure 5. (Harrison S., 1991)

Figure 5. Decompression chamber

3.3.3 Osmotic shock:

The proper functionality of cell's processes usually requires strictly defined chemical conditions. This means e.g. that cell's internal pH or salt concentrations should not deviate significantly from the optimal values. The optimal conditions and ability to withstand suboptimal conditions are species specific. Cells have an ability to actively control the internal conditions but sudden and major changes in cell's surrounding environment might lead to extreme shock which results in cell death and disruption.

Osmotic shock is a technology which can be utilized in biotechnical applications to cause cell lysis. In this technology, cells are first exposed to either high or low salt concentration. Then the conditions are quickly changed to opposite conditions which lead to osmotic pressure and cell

lysis (figure 6). The reason for that is that water quickly flows from low salt concentration conditions towards conditions with high salt concentration. Thus, if the cells are first exposed to high salt concentration solution, water flows into cell after exposure to low salt concentration.

As a result, pressure in cell increases and cell explodes (Stanbury et al. 2016). Conversely, if cell are exposed to high salt concentration (~1 molar solution) after exposure to low concentration, water flows out of the cell which leads to cell disruption.

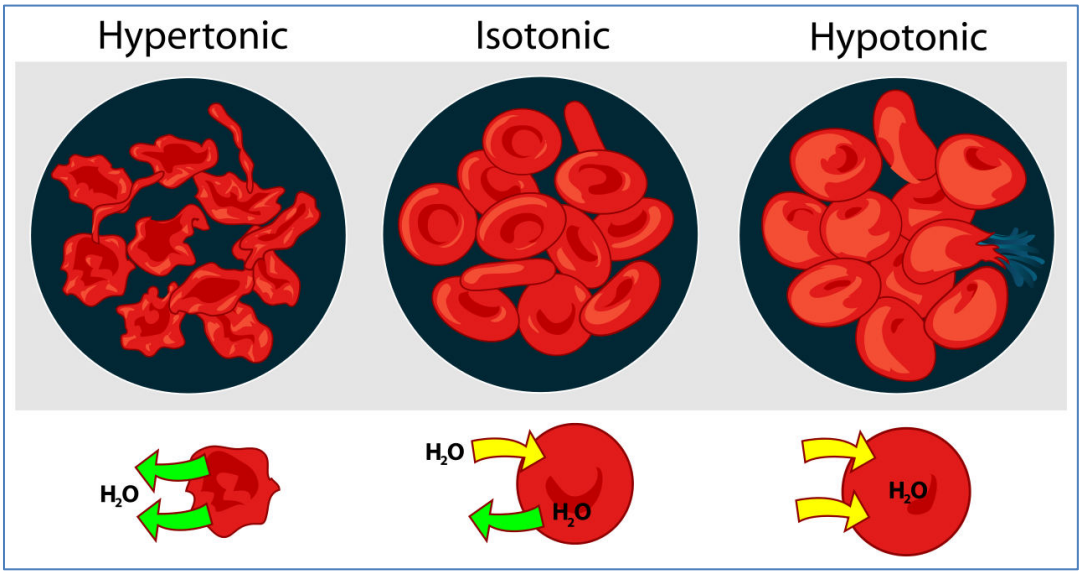


Figure:6: Osmotic shock. Exposure of cells to either high or low salt concentration causes cell disruption.

Osmotic shock is not commonly used method for cell disruption because of its low efficiency. The efficient disruption would commonly require for example enzymatic pre-treatment to weaken the cells. In addition, this technology requires addition of high amounts of salts and water usage is high. Also product may be diluted which increases downstream processing costs.

(Ref: CHEM-E3140 Bioprocess Technology II)

Lecture 19

Non-mechanical chemical and enzymatic methods

3.4. Non-mechanical chemical and enzymatic methods:

In addition to physical and mechanical methods, several chemical methods for cell disruption exist. These methods rely on utilization of chemical substances or enzymes in disruption process. The mechanisms of actions are multiple, but the most widely used methods act by destroying the cell wall by enzymes, osmotic pressure, or by interfering or precipitating cell wall proteins. In

addition, several disruption methods can be combined to achieve desired efficiency. The alternative strategies are reviewed in more detail below.

3.4.1 Detergents:

Detergents that are used for disrupting cells are divided into anionic, cationic and non-ionic detergents. The common thing for all detergents is that they directly damage the cell wall or membrane, and this will lead to release of intracellular content (figure 7). One of the most commonly used anionic detergent is sodium dodecyl sulfate (SDS) which reorganizes the cell membrane by disturbing protein-protein interactions (Thermo Fisher Scientific | Detergents for Cell Lysis and Protein Extraction). Another commonly used compound for cell lysis is Triton X100, which is non-ionic detergent. Its mechanism of action is to solubilize membrane proteins (Harrison 2011). In addition to these chemical compounds, for example cationic detergent ethyl trimethyl ammonium bromide can also be used for cell disruption. It is speculated that it acts on cell membrane lipopolysaccharides and phospholipids (Stanbury et al. 2016).

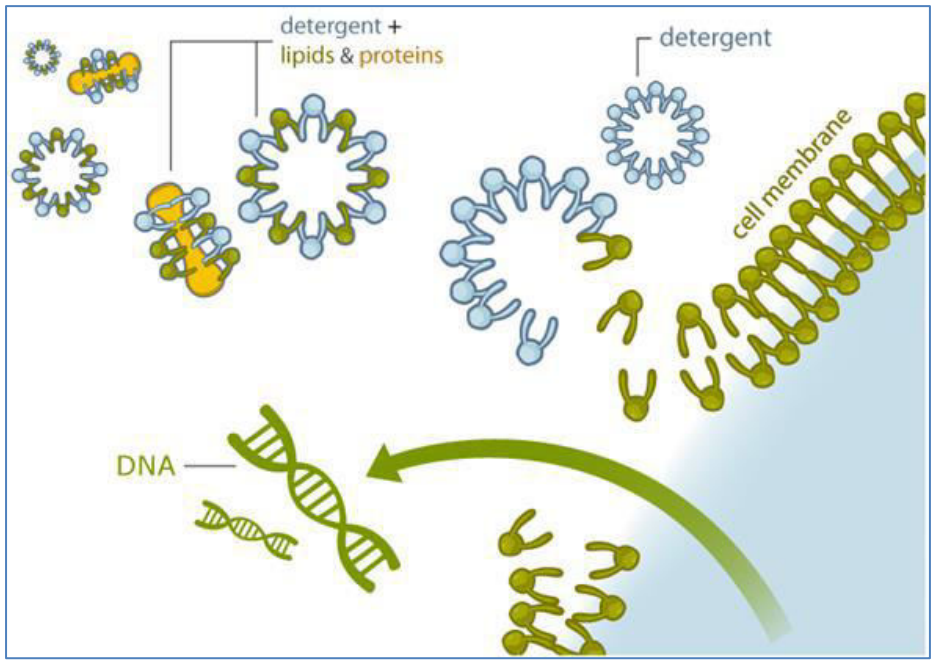


Figure:7: Cell disruption with detergents. Detergents interact with cell membrane compounds which will lead to disassembly of cell membrane.

The disadvantage of using detergents for cell lysis is that many proteins will be denatured in lysis process. Detergents may also disturb subsequent downstream processing steps. Thus additional purification step may be required after cell lysis, which limits their utilization in large scaleprocesses. However, detergents are commonly used for cell lysis in laboratory for example once DNA, RNA or proteins are extracted from cells.

3.4.2. Solvents:

One additional method for chemical cell disruption is the utilization of chemical solvents. Solvents which can be used for cell lysis include for example some alcohols, dimethyl sulfoxide, methyl ethyl ketone or toluene (Stanbury et al. 2016). These solvents extract cell wall's lipid components which leads to release of intracellular components. This method can be used with wide range of production organisms but the problem can be that some proteins are denatured. However, the advantage is that by the choice of solvent, it might be possible to select the relished product. This method is not generally applied in large scale processes.
In addition to solvents, cell lysis can be achieved by hydrolysing the cell wall by alkali compound (pH 10.5-12-5). Disadvantage of this method is that chemical costs for neutralization of alkali are high. In addition, the product may not be stable in alkali conditions.

3.4.3. Enzymes:

Another strategy to achieve cell lysis is to use digestive enzymes which will decompose the microbial cell wall (figure 8). Different cell types and strains have different kind of cell walls and membranes, and thus the used enzyme depends on microbe. For example, lysozyme is commonly used enzyme to digest cell wall of gram positive bacteria. Lysozyme hydrolyzes β-1-4-glucosidic bonds in the peptidoglycan (Crapisi et al. 1993). The cell wall of gram negative bacteria differs from the cell wall of gram positive bacteria so lysozyme is not very efficient in the case of gram negative cell wall.

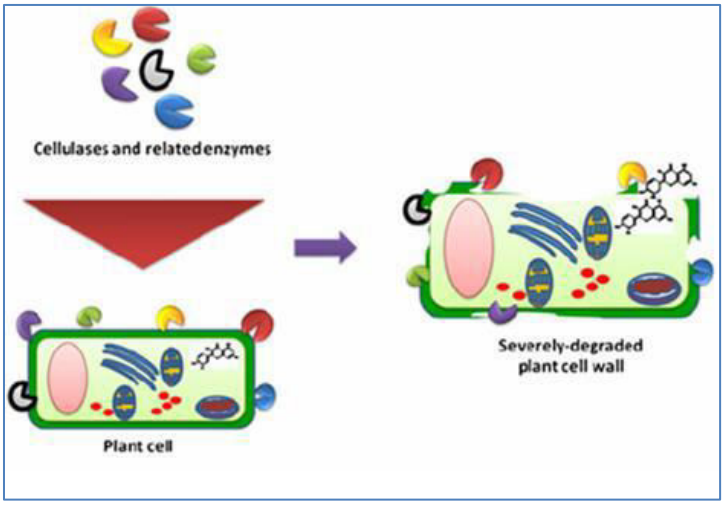


Figure:8: Enzymatic cell disruption concept. Enzymes degrade the cell wall components which will lead to release of intracellular compounds

The cell wall of yeast and fungi differs significantly from the cell wall bacteria. One commonly used enzyme mixture for degradation of cell wall of yeast and fungi isZymolyase. It has for example β-1,3glucanase and β-1,3-glucan laminaripentao-hydrolase activities (Zymolyase | Yeast lytic enzyme). In addition, the enzymes that are commonly used for degradation of cell wall of yeast and fungi include different cellulases, pectinases, xylanases and chitinases.

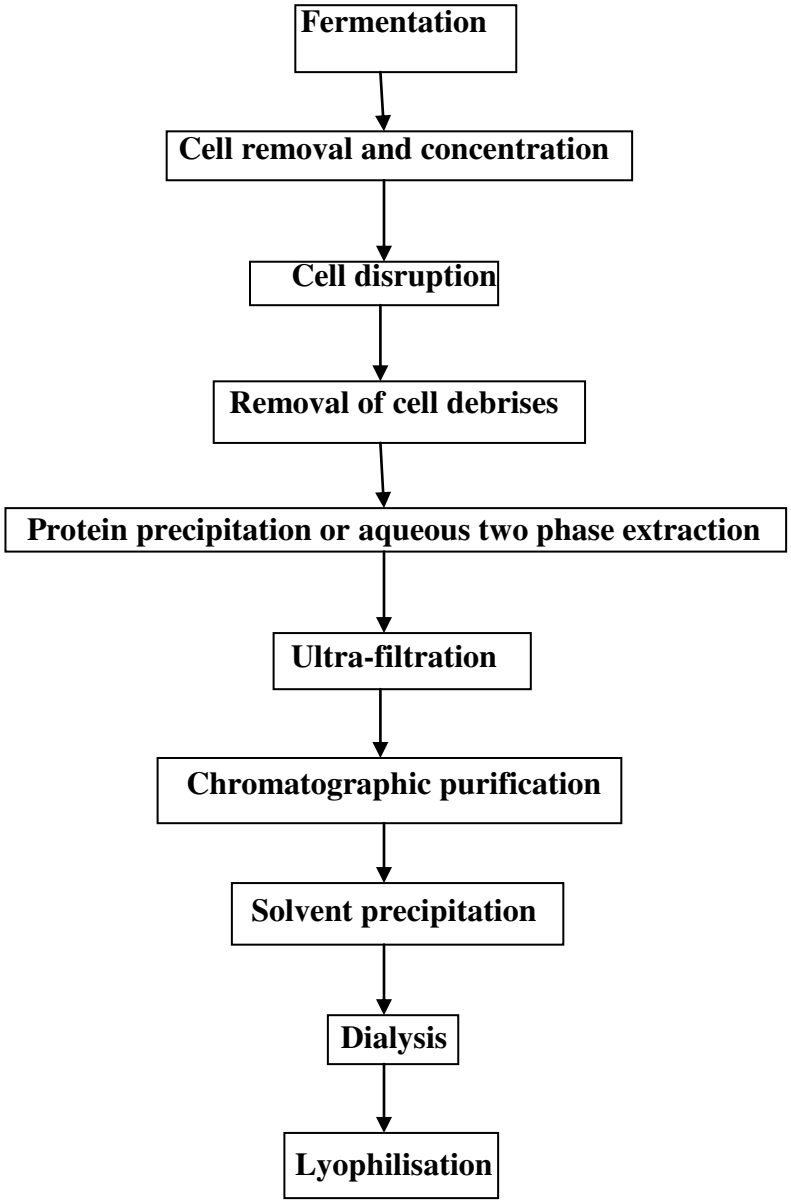
The utilization of enzymes in cell lysis process is one of the gentlest methods (Harrison 2011). However, the enzyme's high price and limited availability limits their utilization in large scale processes. In addition, the added enzyme may complicate downstream processing (e.g. purification). However, these drawbacks could be minimized by immobilization of enzymes (Crapisi et al. 1993).

(Ref: CHEM-E3140 Bioprocess Technology II)

Lecture 20

Recovery and Purification of intracellular enzymes: Overview

3.4. Flow-diagram of Recovery and Purification of intracellular enzymes:



Separation of insoluble particles:
Primary methods are:

- Filtration
- Centrifugation
- Coagulation and Flocculation

Lecture 21

Enzyme Purification Methods: Salting Out

3.5. Enzyme Purification Methods:

Different purification methods of enzymes are:

i. Salting out
ii. Organic solvent precipitation
iii. Dialysis
iv. Reverse osmosis

3.5.1. Salting-Out Method:

Salting out occurs in aqueous solutions of high ionic strength that reduce the molecule's solubility causing certain proteins to precipitate. Ideally, the type of salt being used and the concentration of the salt can be varied to selectively precipitate a the molecule. In reality, salting out is an effective means for initial molecule purification, but lacks the ability for precise isolation of a specific protein.

The Mechanism behind Salting Out

The conformation of large biomolecules *in vivo* is typically controlled by hydrophobic and hydrophilic interactions with the cellular environment. These interactions largely govern the molecule's final conformation by folding in such a way that most hydrophobic functional groups are shielded from the polar cellular environment. To achieve this conformation the molecule folds in such a way that all of the hydrophobic parts of a molecule are aggregated together and the hydrophilic groups are left to interact with the water. In the case of proteins it is the charged amino acids that allow selective salting out to occur. Charged and polar amino acids such as glutamate, lysine, and tyrosine require water molecules to surround them to remain dissolved. In an aqueous environment with a high ionic strength, the water molecules surround the charges of the ions and proteins. At a certain ionic strength, the water molecules are no longer able to support the charges of both the ions and the proteins. The result is the precipitation of the least soluble solute, such as proteins and large organic molecules.

The Hoffmeister Series:

Salting out can be a powerful tool to separate classes of proteins that vary in size, charge, and surface area among other characteristics. One method of controlling the precipitation is the utilize the different effects of various salts and their respective concentrations. A salt's ability to induce selective precipitation is dependent on many interactions with the water and solutes. Research by Franz Hofmeister in the early 20th century organized various anions and cations by their ability to salt out.

The ordering of cations and anions is called the Hoffmeister Series (1). The cations are arranged as follows

$$NH_4^+ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+}$$

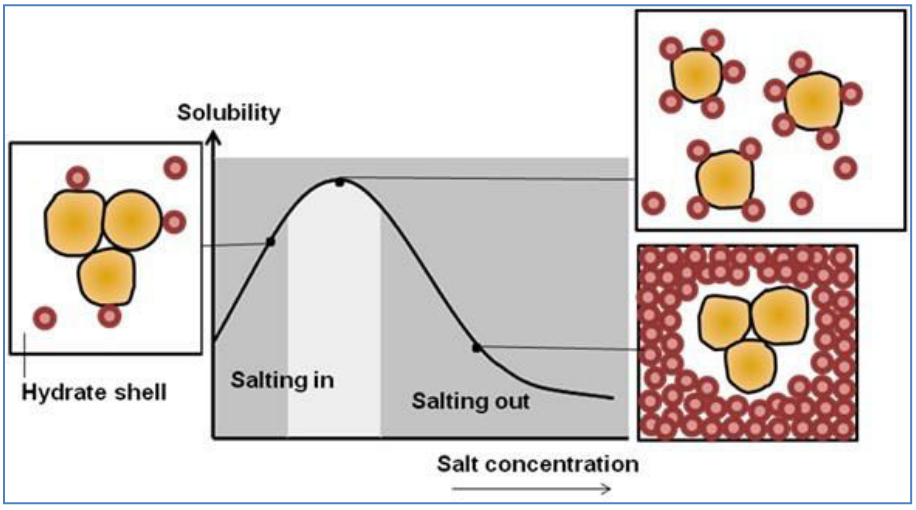
where ammonium has the highest ability to precipitate other proteinacious solutes. Likewise, the order for anions is

$$F^- \geq SO_4^{2-} > H_2PO_4^- > H_3CCOO^- > Cl^- > NO_3^- > Br^- > ClO_3^- > I^- > ClO$$

Between cations and anions in solution the concentration of the anion typically has the greatest effect on protein precipitation.

One of the most commonly used salts is ammonium sulfate, which is typically used because the ions produced in an aqueous solution are very high on the *Hofmeister* series, and their interaction with the protein itself is relatively low. Other ions such as iodide are very good at precipitating proteins, but are not used due to their propensity to denature or modify the protein.

(Ref:https://chem.libretexts.org/Bookshelves/Physical_and_Theoretical_Chemistry_Textbook_Maps/Supplemental_Modules_(Physical_and_Theoretical_Chemistry)/Thermodynamics/Real_(NonIdeal)_Systems/Salting_Out)



Salting-Out Process

Lecture 22

Enzyme Purification Methods: Organic solvent precipitation

3.5.2. Organic solvent precipitation:

Precipitation with organic solvents, such as ethanol and acetone, has been in use for well over a hundred years, but is probably best known for its use in fractionating human serum in the classic work of Cohen and Edsall. Care must be taken to carry out precipitations at very cold temperatures to avoid protein denaturation.

The solubility of protein depends on, among other things, the *dielectric constant* of the solution. In general, solvent molecules with large dielectric constants, e.g. water and dimethylsulphoxide, can stabilize the interaction between themselves and protein molecules and favor the dissolution of protein. On the other hand, organic solvents with small dielectric constants, e.g. acetone and methanol, discourage the dispersion of protein molecules in the media. Thus, the solubility of proteins can be lowered and precipitation can be induced by lowering the effective dielectric constant of the media. This is commonly achieved by adding a water-soluble solvent such as acetone to an aqueous solution of protein. Acetone had the advantage that it is relatively inexpensive and is available in in a pure form with few contaminants that may inhibit or poison the enzyme. It is also frequently used in sterol extraction.

List of Reagents and Instruments

A. Equipment

- Test tubes
- Graduated cylinder
- Pipets
- Balance
- Centrifuge
- Filtration devices

B. Reagents

- Protein solution, 5.0 g/l (albumin, gelatine, casein)
- Enzymes solution, 10 g/l (alpha-amylase, protease)
- Acetone

C. Procedures

i. Precipitation of Protein from Individual Protein Solution:

- Pipet 4 ml of the protein or enzyme solution into a test tube.
- While stirring, add acetone drop-wise to the protein solution from a graduated pipet or a buret until precipitates start to form. Vigorous stirring and slow acetone addition rate will avoid the localized high concentration of acetone.

ii. Effect of Temperature on Enzyme Isolation:

- Perform Step for enzyme solutions 1 at 0ºC and compare the enzymatic activities.

iii. Isolation of Protein Components from a Mixture:

- Repeat the same procedures as in ammonium sulfate precipitation.

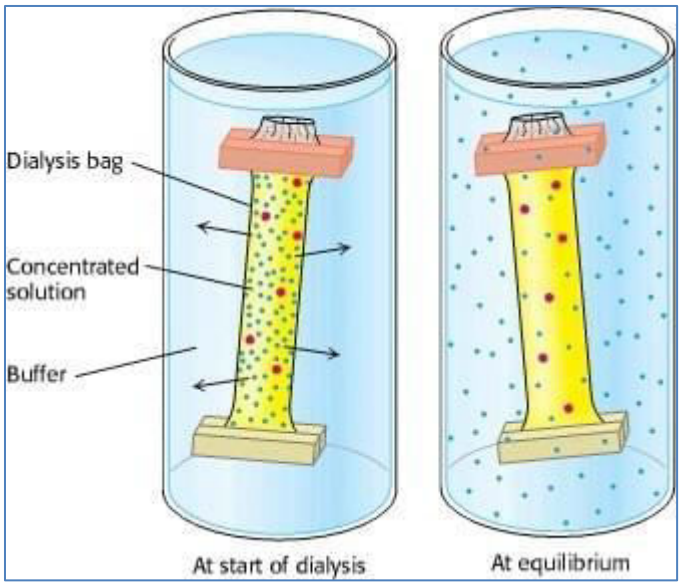
Lecture 23

Enzyme Purification Methods: Dialysis

3.5.3. Dialysis:

In dialysis a semipermeable membrane is used to separate small molecules and protein based upon their size. A dialysis bag made of a semipermeable membrane (cellulose) and has small pores. The bag is filled with a concentrated solution containing proteins. Molecules that are small enough to pass through the pores of the membrane diffuse out of the bag into the buffer solution, or dialysate. Dialysis is sometimes used to change buffers. The molecules go from an area of high concentration to low concentration. When the level of concentration is equal between the bag and the buffer, there is no more net movement of molecules. The bag is taken out and inserted into another buffer, causing the concentration to be higher in the bag relative to the buffer. This causes more diffusion of molecules. This process is repeated several times to ensure that all or most of the unwanted small molecules are removed (usually done overnight). In general, dialysis is not a means of separating proteins, but is a method used to remove small molecules such as salts. At equilibrium, larger molecules that are unable to pass through the membrane remain inside the dialysis bag while much of the small molecules have diffused out.

As the enzymes are relatively large molecules, separation based on the size or mass of molecules favors purification of enzymes, especially the ones with high molecular weight. Dialysis is a commonly used method, where semipermeable membranes are used to remove salts, small organic molecules, and peptides. The process usually needs a large volume of dialysate, the fluid outside the dialysis dag, and a period of hours or days to reach the equilibrium. Countercurrent dialysis cartages can also be used, in which the solution to be dialyzed flow in one direction, and the dialysate in the opposite direction outside of the membrane. Similarly, ultrafiltration membranes, which are made from cellulose acetate or other porous materials, can be used to purify and concentrate an enzyme larger than certain molecular weight. The molecular weight is called the molecular weight cutoff and is available in a large range from different membranes. The ultrafiltration process is usually carried out in a cartridge loaded with the enzyme to be purified. Centrifugal force or vacuum is applied to accelerate the process. Both dialysis and ultrafiltration are quick but somewhat vague on distinguishing the molecular weight, whereas size exclusion chromatography gives fine fractionation from the raw mixture, allowing separation of the desired enzyme from not only small molecules but also other enzymes and proteins. Size exclusion chromatography, also known as gel-filtration chromatography, relies on polymer beads with defined pore sizes that let particles smaller than a certain size into the bead, thus retarding their egress from a column. In general, the smaller the molecule, the slower it comes out of the column. Size exclusion resins are relatively “stiff” and can be used in high pressure columns at higher flow rates, which shortens the separation time. Other factors including the pore size, protein shape, column volumes, and ionic strength of the eluent could also change the result of purification.



The scheme of dialysis: Enzyme molecules (red dots) are retained in the dialysis bag and separated from other smaller molecules (blue dots)

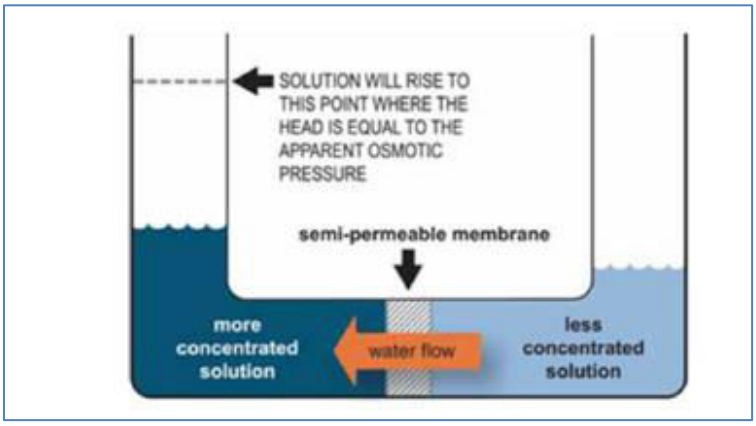
(**Reference:** Berg JM, Tymoczko JL, Stryer L. Biochemistry. 5th edition. New York: W H Freeman; 2002. Section 4.1)

Lecture 24

Enzyme Purification Methods: Reverse Osmosis

3.5.4. Reverse Osmosis:

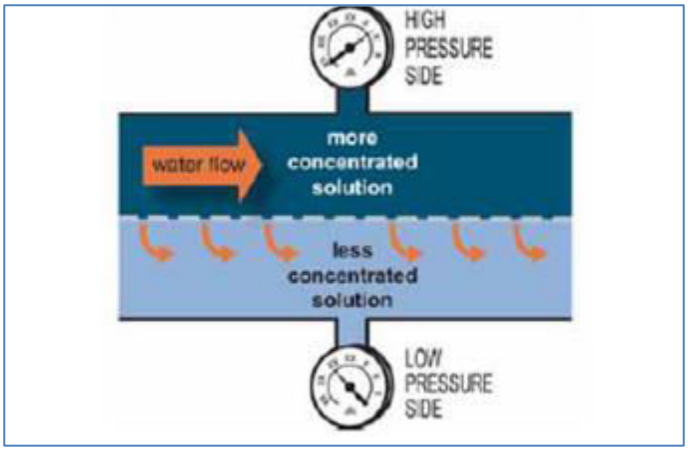
In order to understand the Reverse Osmosis (RO), it first requires to understand the concept of most common natural phenomena known as osmosis. Osmosis is general and important process in the nature, which can be easily defined by water passing from less concentrated solution towards the highly concentrated solution through semipermeable barrier or membrane. The transfer carries on occurring until the both solution gain equal concentration. A figure below describes simple dynamics of the osmosis process.



Natural Osmosis Process

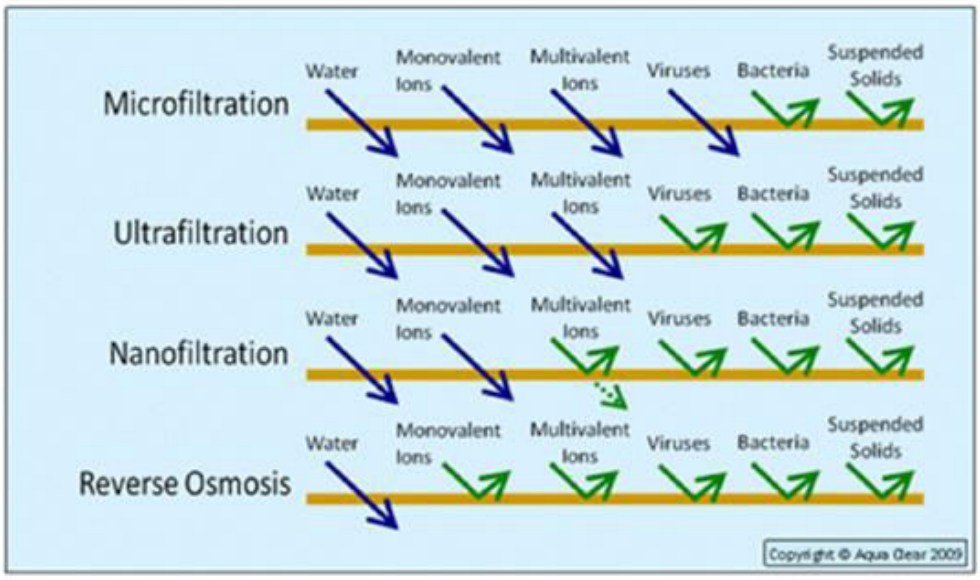
RO is completely alter process of osmosis. Osmosis occurs naturally without requiring external energy, whereas RO requires to apply external energy/pressure using a high pressure pump on the side of the highly concentrated solution. At osmotic pressure, the flow between two solutions will be ceased whereas applying pressure or energy greater than osmotic pressure reverse the flow form highly concentrated solution towards the less concentrated solution . The amount of pressure required depends on the salt concentration of the feed water. The higher the

concentration of the feed water, the more pressure is required to overcome the osmotic pressure. A simple schematic of reverse osmosis is given below.



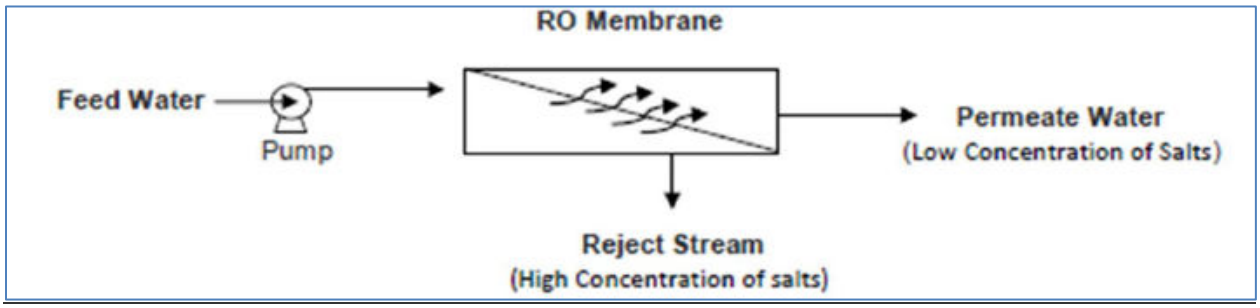
Schematic diagram of RO process

Reverse osmosis is membrane filtration technique. Within the membrane filtration, there are many other technologies are also well known along Reverse Osmosis :Nanofiltration (NF) , Ultrafiltration (UF) and Microfiltration (MF). Membrane filtration techniques are well employed in different fields because of their efficiency, process maturity and cost effectiveness mainly. The picture below clarifies the possible components that can be separated using these technologies.



Comparison chart of different filtration various membrane filtration technologies

RO is very effective techniques for treating water. According to the literature, RO is capable of removing up to 99% of the dissolved salts (ions), particles, colloids, organics, bacteria and pyrogens from the feed water. The membrane within the RO rejects contaminants based on their size and charge. Molecules with molecular weight greater than 200 are likely to be rejected by the fully functioning RO system. Besides, the greater the ionic charge of contaminants has higher chances of getting stuck in membrane. However, the gases are not removed by the RO because gases such as CO2 is not highly ionized while in the solution and have very low molecular weight.



Operation of RO water purification system

In general, feed water is pumped into a Reverse Osmosis (RO) system which ends up with two types of liquid coming out of the RO system: the permeate and the reject stream. The reject stream, also called concentrate or brine stream contains the contaminants, as they are not allowed to pass through the membrane. The water that makes it out through the membrane, with very little contaminants, is called permeate or product water.

(Ref: CHEM-E3140 – Bioprocess technology II Dialysis and reverse osmosis, by Emilie Baroux, AnikNath, Sirkku Whitney)

Module VI 8L

Lecture 25

Application of Enzymes for Production in Biochemical and Food Processing Industries

4.1. Applications of microbial enzymes in food industry:

Microbial enzyme	Application
α-Amylase	• Baking, brewing, starch liquefaction • Bread quality improvement • Rice cakes • Clarification of fruit juice
Glucoamylase	• Beer production • Bread quality improvement • High glucose and high fructose syrups
Protease	• Brewing • Meat tenderization • Coagulation of milk • Bread quality improvement
Lactase (β-galactosidase)	• Lactose intolerance reduction in people • Prebiotic food ingredients
Lipase	• Cheese flavour development • Cheddar cheese production
Phospholipase	• Cheese flavour development • Production of lipolyzed milk fat
Esterase	• Enhancement of flavour and fragrance in fruit juice • De-esterification of dietary fibre • Production of short chain flavour esters
Cellulase	• Animal feed • Clarification of fruit juice
Xylanase	• Clarification of fruit juice • Beer quality improvement
Pectinase	• Clarification of fruit juice
Glucose oxidase	• Food shelf life improvement

	• Food flavour improvement
Laccase	• Polyphenol removal from wine • Baking
Catalase	• Food preservation (with glucose oxidase) • Removal of hydrogen peroxide from milk prior to cheese production
Peroxidase	• Development of flavour, colour and nutritional quality of food
α-Acetolactate dehydrogenase	• Shortening maturation of beer
Asparaginase	• Reduction of formation of acrylamide during baking
Debittering enzymes - naringinase	• Removal of bitter taste in fruit juice • Wine aroma enhancement

Lecture 26

Enzymes Used in Dairy Industries

4.1. 1.Enzymes Used in Dairy Industries:

In the dairy industry, some enzymes are required for the production of cheeses, yogurt, and other dairy products, while others are used in a more specialized fashion to improve texture or flavor. Five of the more common types of enzymes and their role in the dairy industry are described below.

Rennet

Milk contains proteins, specifically caseins, that maintain its liquid form. Proteases are enzymes that are added to milk during cheese production, to hydrolyze caseins, specifically kappa casein, which stabilizes micelle formation preventing coagulation. Rennet and rennin are general terms for any enzyme used to coagulate milk. Technically rennet is also the term for the lining of a calf's fourth stomach.

The most common enzyme isolated from rennet is chymosin. Chymosin can also be obtained from several other animals, microbial or vegetable sources, but indigenous microbial chymosin (from fungi or bacteria) is ineffective for making cheddar and other hard cheeses.

Limited supplies of calf rennet have prompted genetic engineering of microbial chymosin by cloning calf prochymosin genes into bacteria. Bioengineered chymosin may be involved in the production of up to 70% of cheese products. While the use of bioengineered enzymes spares the lives of calves, it presents ethics issues for those opposed to eating foods prepared with GEMs.

Lactalbumin and Lactoglobulin

Milk contains a number of different types of proteins, in addition to the caseins. Cow milk also contains whey proteins such as lactalbumin and lactoglobulin. The denaturing of these whey proteins, using proteases, results in a creamier yogurt product. Destruction of whey proteins is also essential for cheese production.

During the production of soft cheeses, whey is separated from the milk after curdling and may be sold as a nutrient supplement for bodybuilding, weight loss, and lowing blood pressure, among other things. There have even been reports of dietary whey for cancer therapies, and having a role in the induction of insulin production for those with Type 2 diabetes.

Proteases are used to produce hydrolyzed whey protein, which is whey protein broken down into shorter polypeptide sequences. Hydrolyzed whey is less likely to cause allergic reactions and is used to prepare supplements for infant formulas and medical uses.

Lactase

Lactase is a glycoside hydrolase enzyme that cuts lactose into its constituent sugars, galactose, and glucose. Without sufficient production of lactase enzyme in the small intestine, humans become lactose intolerant, resulting in discomfort (cramps, gas, and diarrhea) in the digestive tract upon ingestion of milk products.

Lactase is used commercially to prepare lactose-free products, particularly milk, for such individuals. It is also used in the preparation of ice cream, to make a creamier and sweeter tasting product. Lactase is usually prepared from *Kluyveromyces* sp. of yeast and *Aspergillus* sp. of fungi.

Catalase

The enzyme Catalase has found limited use in one particular area of cheese production. Hydrogen peroxide is a potent oxidizer and toxic to cells. It is used instead of pasteurization, when making certain cheeses such as Swiss, in order to preserve natural milk enzymes that are beneficial to the end product and flavor development of the cheese.

These enzymes would be destroyed by the high heat of pasteurization. However, residues of hydrogen peroxide in the milk will inhibit the bacterial cultures that are required for the actual cheese production, so all traces of it must be removed. Catalase enzymes are typically obtained from bovine livers or microbial sources and are added to convert the hydrogen peroxide to water and molecular oxygen.

Lipases

Lipases are used to break down milk fats and give characteristic flavors to cheeses. Stronger flavored cheeses, for example, the Italian cheese, Romano, are prepared using lipases. The flavor comes from the free fatty acids produced when milk fats are hydrolyzed. Animal lipases are obtained from kid, calf, and lamb, while microbial lipase is derived by fermentation with the fungal species *Mucormeihei*.

Although microbial lipases are available for cheese-making, they are less specific in what fats they hydrolyze, while the animal enzymes are more partial to short and medium-length fats. Hydrolysis of the shorter fats is preferred because it results in the desirable taste of many kinds of cheese. Hydrolysis of the longer chain fatty acids can result in either soapiness or no flavor at all.

(Ref: https://www.thebalance.com/enzymes-used-in-the-dairy-industry-375519)

Lecture 27

Enzymes Used in Bakery Industries

4.1.2. Enzymes Used in Bakery Industries:

Enzyme	Effect
Amylase	Maximises the fermentation process to obtain an even crumb structure and a high loaf volume
Maltogenic alpha-amylases	Improves shelf-life of bread and cakes
Glucose oxidase	Oxidative reaction with gluten to make weak doughs stronger, drier and more elastic
Lipase	Modifies the natural lipids in flour to strengthen the dough

Lipoxygenase	Bleaching and strengthening dough
Xylanase	Dough conditioning. Easier dough handling and improved crumb structure
Protease	Weakens the gluten to give plastic properties required in doughs for biscuits
Asparginase	Reduces the amount of acrylamid formed during baking

Some of the bread properties that can be improved using industrial enzymes

Flour supplementation

Malt flour and malt extract can be used as enzyme supplements because malt is rich in alpha-amylases. Commercial malt preparations can differ widely in their enzyme activity, whereas an industrial enzyme is supplied with a standardised activity.

The alpha-amylases degrade the damaged starch in wheat flour into small dextrins, which allows yeast to work continuously during dough fermentation, proofing and the early stage of baking. The result is improved bread volume and crumb texture. In addition, the small oligosaccharides and sugars such as glucose and maltose produced by these enzymes enhance the Maillard reactions responsible for the browning of the crust and the development of an attractive baked flavour.

Bread and cake staling is responsible for significant financial loss for both consumers and producers. For instance, every year in the USA bread worth more than USD 1 billion is discarded. However the main saving on prolonging the shelf life is actually savings in transportation and fuel costs due to a more efficient distribution. Staling is associated with a loss of freshness in terms of increased crumb firmness, decreased crumb elasticity and loss of moistness.

Staling is believed to be due to changes in starch structure during storage. When the starch granules revert from a soluble to an insoluble form, they lose their flexibility; the crumb becomes hard and brittle. For decades, emulsifiers have been used as anti-staling agents. However, they actually have a limited anti-staling effect and are subject to special labelling rules.

Dough conditioning

Flour contains 2.5-3.5% non-starch polysaccharides, which are large polymers (mainly pentosans) that play an important role in bread quality due to their water absorption capability and interactions with gluten. Although the true mechanism of hemicellulase, pentosanase or

xylanase in bread-making has not been clearly demonstrated, it is well known that the addition of certain types of pentosanases or xylanases at the correct dosage can improve dough machinability yielding a more flexible, easier-to-handle dough. Consequently, the dough is more stable and gives better ovenspring during baking, resulting in a larger volume and improved crumb texture.

Normal wheat flour contains 1-1.5% lipids, both polar and non-polar. Some of these lipids, especially the polar lipids such as phosphorlipids and galactolipids are able to stabilise the air bubbles in the gluten matrix. The addition of functional lipases modifies the natural flour lipids so they become better at stabilizing the dough. This ensures a more stable dough in case of over-fermentation, a larger loaf volume, and significantly improved crumb structure. Because of the more uniform and smaller crumb cells, the crumb texture is silkier and the crumb colour appears to be whiter. It also reduces the need for addition of emulsifiers like DATEM and SSL that otherwise are commonly added to dough in order to stabilise it. This in turn means that emulsifiers can be removed from the label.

Glucose oxidase and fungal amylase (right-hand loaf) were used to replace bromate in Maraquetta (South American bread)

Chemical oxidants such as bromates, azodicarbonamide and ascorbic acid have been widely used to strengthen the gluten when making bread. As an alternative, oxidases such as glucose oxidase can partially replace the use of these chemical oxidants and achieve better bread quality.

As shown in above, glucose oxidase and fungal alpha-amylase can be used not only to replace bromate but also to give a greater bread volume.

The synergistic effects of enzymes

Each of the enzymes mentioned above has its own specific substrate in wheat flour dough. For example, lipases work on the lipids, xylanase works on the pentosans, and amylases work on the starch. Because the interaction of these substrates in dough and bread is rather complex, the use of enzyme combinations can have synergistic effects that are not seen if only one enzyme is

used, not even at high dosages. Quite often an over dosage of enzymes will have a detrimental effect on either the dough or the bread. For instance, an overdose of fungal alpha-amylase or hemicellulase / xylanase may result in a dough that is too sticky to be handled by the baker or baking equipment. It is therefore beneficial for some types of bread formulation to use a combination of lower dosages of alpha-amylase and xylanase with low dosages of lipase or glucose oxidase to achieve optimum dough consistency stability and bread quality. Another example is to use maltogenic alpha-amylase in combination with fungal alpha-amylases and xylanase or lipase to secure optimum crumb softness as well as optimum bread quality in terms of crumb structure, bread volume, etc.

Reduction of acrylamide content in food products

During recent years it has been shown that the amount of the potentially carcinogenic substance acrylamide is relatively high in a number of cereal and potato based products like biscuits, crackers, crisp bread, French fries and potato crisps. This is a substance that is formed at high temperatures when the amino acid asparagine reacts with a reducing sugar like glucose. To meet this issue the enzyme asparaginase have been developed in order to reduce the formation of acrylamide. Asparaginase converts asparagine to aspartic acid which does not take part in the formation of acrylamide. The use of an asparginase is able to reduce the formation of acrylamide with up to 90%.

Lecture 28

Application of Enzymes in Brewing Industries

4.1.3. Application of Enzymes in Brewing Industries:

Beer and wine, both beverages are produced by yeast fermentation of sugars. Wine is based on grapes, and beer is traditionally based on barley. The matured grapes already contain the sugars needed for the fermentation, while barley contain starch that has to be broken down to fermentable sugars before the yeast can make alcohol. Therefore, traditional brewing contains and extra step compared with wine-making, namely malting in which enzymes needed for the degradation of starch into fermentable sugars are produced.

The brewing process

Traditionally, beer is produced by mixing crushed barley malt and hot water in a mash copper to perform the mashing. Besides malt, other starchy cereals such as maize, sorghum, rice and barley, or pure starch itself, can be added to the mash. These are known as adjuncts.

The standard mashing for pilsner type beer consists of several temperature steps, each favouring different malt enzyme activities. The lowest temperature (45 ºC) is the optimal temperature for cell wall degrading enzymes, β-glucanases. The proteases works best at 52 ºC, the β-amylase best at 63 ºC and the α-amylase at 72°C. The last step in the mashing is inactivation of the enzymes at 78 ºC (Figure 3).

If β-glucan and protein are properly broken down during malting, single temperature mashing at 65-71°C has shown to be sufficient, as in the case of traditional ale brewing.

During mashing the starch is degraded to dextrin and fermentable sugars. α-amylase liquefy the gelatinized starch by hydrolysis of the α-1,4 linkages at random. β-amylases are exo-enzymes which attack the liquefied starch chains resulting in successive removal of maltose units from the non-reducing end.

After mashing, the mash is sieved in a lautertun or on a mash filter. The resulting liquid, known as sweet wort, is then transferred to the copper, where it is boiled with hops. The hopped wort is cooled and transferred to the fermentation vessels, where yeast is added. In normal wort 2/3 of the carbohydrates are fermentable sugars. After fermentation, the so-called ‘green beer' is matured before final filtration and bottling

Enzymes at work

Quality and supply constraints on malt, and doubling of malt prices have given increased interest for enzyme solutions in 2007 and 2008. Many breweries has run programs within the last two years in order to increase efficiency and optimize raw material usage, and many of them have focused on commercial enzymes to shorten the production time, increase capacity, and to allow use of raw material alternative to malt. Three important examples are mentioned:

Exchanging part of the malt with barley has been popular because using barley in combination with commercial enzymes gives the same beer quality as with malt.

Introducing a higher content of starch hydrolysing enzymes offer the possibilities of producing "light beer" also called "low calorie beer".

An enzyme solution for diacetyl control after fermentation improves vessel utilization, save energy and ensures a high beer quality after a reduced maturation time.

Operation	Enzymes	Enzyme action	Function
Decoction vessel (cereal cooker)	α-amylase	Hydrolyse starch	Adjunct* liquefaction. Reduce viscosity
	β-glucanase	Hydrolyseglucans.	Aid the filtration.
Mashing	α-amylase	Hydrolyse starch.	Malt improvement.
	Amyloglucosidase	Increase glucose content.	Increase % fermentable sugar in "light" beer.
	Debranching enzyme	Hydrolyse α-1,6 branch points of starch.	Secures maximum fermentability of the wort.
	Proteases	Increase soluble protein, and free amino- nitrogen (FAN).	Malt improvement Improved yeast growth.
	β-glucanase	Hydrolyseglucans.	Improve wort separation.
	Pentosanase/xylanase	Hydrolysepentosans of malt, barley, wheat.	Improve extraction and beer filtration.
Fermentation	Fungal α-amylase	Increase maltose and glucose content.	Increase % fermentable sugar in "light" beer.
	β-glucanase	Hydrolyze glucans.	Reduce viscosity and aid filtration.
	α-acetolactate-decarboxylase (ALDC)	Converts α-acetolactate to ace-	Decrease fermentation

		toindirectly.	time by avoiding formation of diacetyl.
Conditioning tank	Protease	Modify protein-polyphenolic compounds.	Reduce the chill haze formed in beer.
* Adjunct is starchy cereals such as maize, rice, wheat, sorghum, barley or pure starch materials added to the mash.			

Steps of the brewing operations where microbial enzymes are used

Lecture 29

Enzymes used in Fruit Juice Industries

4.1.4. Enzymes used in Fruit Juice Industries:

Juice Enzymes

Enzymes	Benefits
Pectinase used for maceration of a wide variety of fruits and vegetables, prior to extraction by decanter centrifuge	· Increases juice yields · Faster extraction by decanter centrifuge · Increases sugar and acid extraction · Color enhancement · Effortless clarification and filtration · Increases processing capacity
Carbohydrase enzyme used in the mash treatment of fruit and vegetables prior to juice extraction by decanter centrifuge	· Increases juice yields · Easier and more rapid juice extraction · Increases sugar (Brix) and acid extraction · Improves juice color and aroma · Faster solids separation and concentration · Increases processing capacity
Pectinase used to depectinize a wide variety of fruit juices at their natural pH	· Provides complete depectinization · Speeds clarification and filtration · Reduces viscosity · Increases evaporator output · Reduces cost of packaging · Produces clear juices and concentrates
Acid protease used to stabilize and eliminate post-boiling haze	· Reduces protein in juices and concentrates
Glucoamylase used for removal of starch from under ripe fruit prior to filtration	· Excellent thermal and pH stability

Lecture 30

Enzyme Immobilization: Overview and Methods of immobilization

4.2. Enzyme Immobilization:

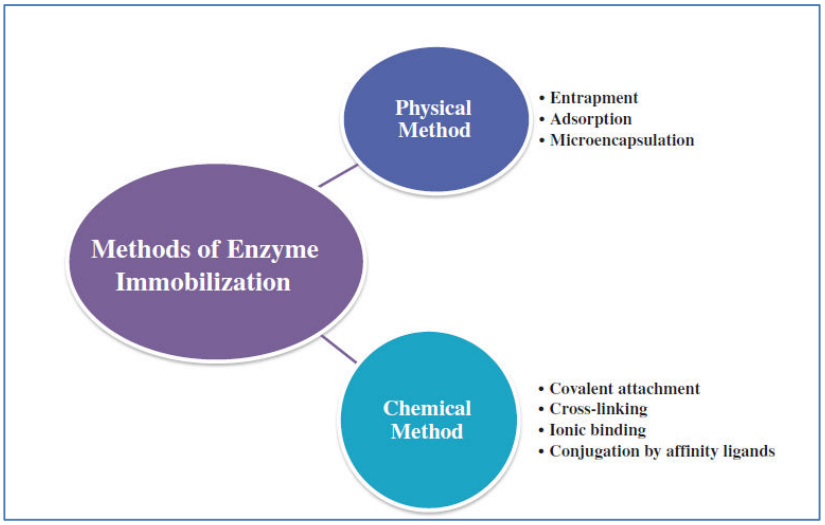
An immobilized enzyme is an enzyme attached to an inert, insoluble material—such as calcium alginate (produced by reacting a mixture of sodium alginate solution and enzyme solution with calcium chloride). This can provide increased resistance to changes in conditions such

as pH or temperature. It also lets enzymes be held in place throughout the reaction, following which they are easily separated from the products and may be used again - a far more efficient process and so is widely used in industry for enzyme catalyzed reactions. An alternative to enzyme immobilization is whole cell immobilization.

4.2.1. Methods of Enzyme Immobilization

4.2.1.1. Physical Method

Enzyme attachment onto different matrices *via* physical forces involving van der Waals forces, hydrophobic interactions and hydrogen bonding. The process is reversible in nature by controlling physicochemical parameters.



Overview on the techniques being used for enzyme immobilization

(i) Entrapment:

This involves cross-linking of the enzyme to a polymer (polyacrylamide, alginate etc.) in every direction, covering almost every side chain present on the surface of the enzyme by physical entrapment within the polymer lattice. It allows permeation of appropriately sized substrate and release of product molecules, which ensures continuous transformation. However, this method can only be used in a limited number of enzymes. This method has several advantages: simplicity, no change in intrinsic enzyme properties, involves no chemical modification, minimal enzyme requirement and matrices are available in various shapes. The various disadvantages of this method are; enzyme leakage, only small sized substrate/products can be used, requires

delicate balance between mechanical properties of the matrix and its effect on enzyme activity and presence of diffusional constraints.

(ii) Adsorption:

Enzyme is attached to the support material by non-covalent linkages including ionic or hydrophobic interactions, hydrogen bonding, and van der Waals forces without any pre-activation of support. The matrices used are either organic or inorganic in nature, viz. ceramic, alumina, activated carbon, kaolinite, bentonite, porous glass, chitosan, dextran, gelatin, cellulose, starch. The immobilization method involves optimization of variables including pH, temperature, nature of the solvent, ionic strength, concentration of enzyme and adsorbent. Here, the enzyme is directly added to the surface (active adsorbent) without the removal of any non-adsorbed enzyme during washing. The method is simple and mild with a vast variety of carriers helpful for simultaneous purification as well as enzyme immobilization (e.g. Asparginase on CM-cellulose) without any conformational change. However, it involves intensive optimization due to the involvement of a number of factors that play a role in enzyme desorption following slight changes in its micro-environment (e.g., pH, temperature, solvent, ionic strength and high substrate concentrations).

(iii) Microencapsulation:

Enzymes are immobilized by enclosing them within spherical semi-permeable polymer membranes with controlled porosity (1–100 μm) . Semi-permeable membranes can either be permanent or nonpermanent membranes based on the constituents. Permanent membranes are made of cellulose nitrate and polystyrene while non-permanent membranes are made of liquid surfactant. These membranes are also used in the encapsulation of dyes, drugs, and other chemicals. Enzymes immobilized by encapsulation have extremely large surface areas due to which they have higher catalytic efficiency. However, there are several reports on occasional inactivation of the enzyme; despite presence of high enzyme concentration.

4. 2.1.2 Chemical Method:

This involves attachment of enzymes onto different matrices using covalent or ionic bonds and the process is irreversible.

(i) Covalent attachment:

The enzyme is attached to the matrix by means of covalent bonds (diazotation, amino bond, Schiff's base formation, amidation reactions, thiol-disulfide, peptide bond and alkylation reactions). Enzyme molecules are attached either directly to the reactive groups (e.g., hydroxyl,

amide, amino, carboxyl groups) present on the matrix or by a spacer arm, which is artificially attached to the matrix through various chemical reactions (e.g., diazotization, schiff base, imine bond formation). Matrices commonly used are either natural (e.g., glass, Sephadex, Agarose, Sepharose) or synthetic (e.g., acrylamide, methacrylic acid, styrene). The selection of a particular matrix depends on its cost, availability, binding capacity, hydrophilicity, structural rigidity and durability during various applications. This method of immobilization involves non-essential amino acids (other than active site groups) leading to minimal conformational changes. It helps to promote the higher resistance of immobilized enzymes towards extreme physical and chemical conditions (e.g., temperature, denaturants, organic solvents).

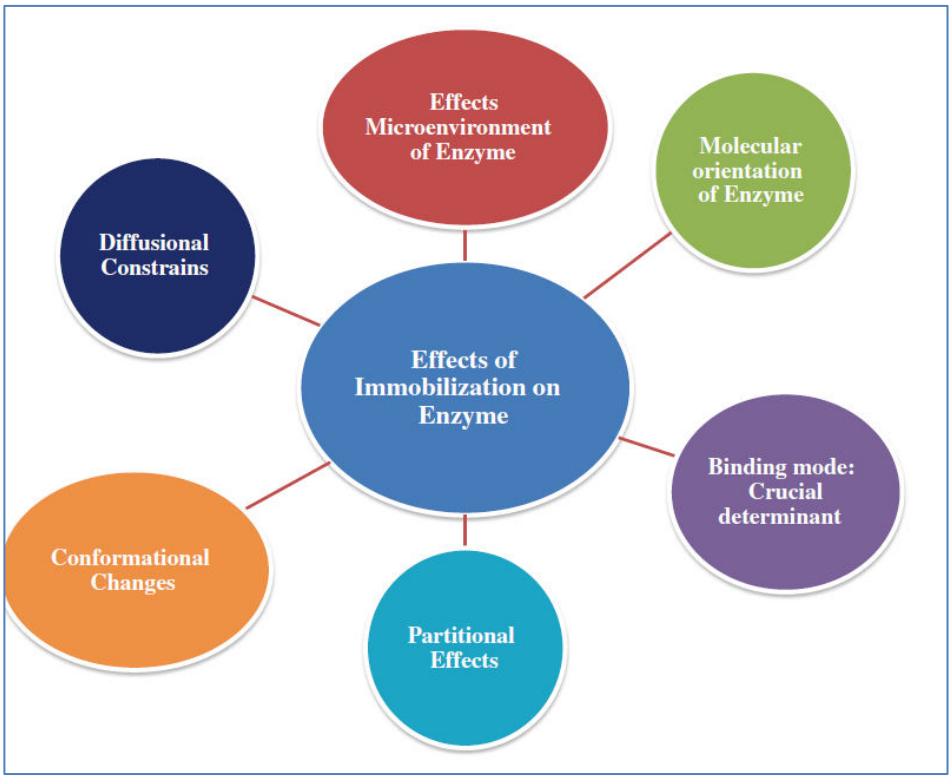
(ii) Conjugation by affinity ligands:

Attachment of the enzyme to the matrix using specific ligands; viz, his-tag on enzyme to a metal-containing matrix, lectin-containing domain to carbohydrate moieties present on the matrix or sometimes substrate-mimicking chemical compounds are also used as ligands. In some cases, ligands are naturally present on the enzyme, while in other cases they are attached artificially by fusing a nucleotide sequence corresponding to the tag with the DNA encoding a polypeptide of the given enzyme. This method of immobilization leads to minimal changes in the conformation of enzyme, with high stability and catalytic efficiency of the immobilized enzyme due to non-involvement of active site residues and higher immobilization efficiency due to the presence of high densities of ligands on the matrix. This method is not only useful for enzyme immobilization but also for several proteins including antibodies, cytokines, streptavidin etc.

Lecture 31

Properties of Immobilized Enzymes

4.2.2. Properties of Immobilized Enzymes:



Effects on enzymes being immobilized on various matrices by either method of immobilization

4.2.3. Matrices for Enzyme Immobilization:

(a) Surface-Bound Enzymes: The physical and chemical properties of the matrices used for enzyme immobilization are very important as they are major governing factors of chemical, biochemical, mechanical and kinetic properties of immobilized enzymes. The matrix can be biopolymer, synthetic organic polymer, hydrogels, smart polymer or inorganic solid.

- Biopolymers: cellulose, starch, agarose, chitosan, and proteins such as gelatin and albumin
- Synthetic organic polymers: Eupergit-C (acrylic resins), Sepa beads FP-EP, Amberlite XAD-7 (porous acrylic resins) etc
- Hydrogels: PVA (polyvinyl alcohol) hydrogel is the most commonly used matrix for enzyme immobilization
- Inorganic solids: alumina, silica, zeolites and mesoporoussilicas (MCM-41, and SBA-15)

- Smart Polymer: The most studied example of smart polymer is thermostable biocompatible polymer [poly-N-isopropylacrylamide (polyNIPAM)].

(b) Entrapment: Enzymes can be immobilized by enclosing them inside the matrices. Sol-gel is a metal alkoxides that has been used for the entrapment of several enzymes. Enzyme immobilization into silica sol-gel is prepared by hydrolytic polymerization of tetraethoxysilane followed by drying. The immobilization method involves drying, which is the determining factor in the morphology of sol-gel.

(Ref: Basics of Enzyme Immobilization)

Lecture 32

Advantages, Disadvantages and Application of Immobilized Enzymes

4.2.4. Advantages of immobilized enzymes:

a. Stable and more efficient in function.

b. Can be reused again and again.

c. Products are enzyme-free.

d. Ideal for multi-enzyme reaction systems.

e. Control of enzyme function is easy.

f. Suitable for industrial and medical use.

g. Minimize effluent disposal problems.

4.2.5. Disadvantages of immobilized enzymes:

a. The possibility of loss of biological activity of an enzyme during immobilization or while it is in use.

b. Immobilization is an expensive affair often requiring sophisticated equipment.

4.2.6. Applications of Immobilized Enzymes:

A selected list of important immobilized enzymes and their industrial applications is given in Table 21.6. Some details on the manufacture of L-amino acids and high fructose syrup are given hereunder.

TABLE 21.6 A selected list of important immobilized enzymes and their industrial applications

Immobilized enzyme	*Application(s)*
Aminoacylase	Production of L-amino acids from D, L-acyl amino acids
Glucose isomerase	Production of high fructose syrup from glucose (or starch)
Amylase	Production of glucose from starch
Invertase	Splitting of sucrose to glucose and fructose
β-Galactosidase	Splitting of lactose to glucose and galactose
Penicillin acylase	Commercial production of semi-synthetic penicillins
Aspartase	Production of aspartic acid from fumaric acid
Fumarase	Synthesis of malic acid from fumaric acid
Histidine ammonia lyase	Production of urocanic acid from histidine
Ribonuclease	Synthesis of nucleotides from RNA
Nitrilase	Production of acrylamide from acrylonitrile

Production of L-Amino Acids:

L-Amino acids (and not D-amino acids) are very important for use in food and feed supplements and medical purposes. The chemical methods employed for their production result in a racemic mixture of D- and L-amino acids. They can be acylated to form D, L-acyl amino acids. The immobilized enzyme aminoacylase (frequently immobilized on DEAE sephadex) can selectively hydrolyse D, L-acyl amino acids to produce L-amino acids.

$$\text{D, L-Acyl amino acids} \xrightarrow{\text{Aminoacylase}} \text{L-Amino acids} + \text{D, L-Acyl amino acids}$$

The free L-amino acids can separated from the un-hydrolysed D-acyl amino acids. The latter can be recemized to D, L-acyl amino acids and recycled through the enzyme reactor containing immobilized aminoacylase. Huge quantities of L-methionine, L-phenylalanine L-tryptophan and L-valine are produced worldwide by this approach.

Production of High Fructose Syrup:

Fructose is the sweetest among the monosaccharide's, and has twice the sweetening strength of sucrose. Glucose is about 75% as sweet as sucrose. Therefore, glucose (the most abundant monosaccharide) cannot be a good substitute for sucrose for sweetening. Thus, there is a great demand for fructose which is very sweet, but has the same calorific value as that of glucose or sucrose.

High fructose syrup (HFS) contains approximately equivalent amounts of glucose and fructose. HFS is almost similar to sucrose from nutritional point of view. HFS is a good substitute for sugar in the preparation of soft drinks, processed foods and baking.

High fructose syrup can be produced from glucose by employing an immobilized enzyme glucose isomerase. The starch containing raw materials (wheat, potato, corn) are subjected to hydrolysis to produce glucose. Glucose isomerase then isomerizes glucose to fructose (Fig. 21.11). The product formed is HFS containing about 50% fructose. (Note: Some authors use the term high fructose corn syrup i.e. HFCS in place of HFS).

Table 21.7 Selected examples of immobilized enzymes used in analytical biochemistry

Immobilized enzyme	*Substance assayed*
Glucose oxidase	Glucose
Urease	Urea
Cholesterol oxidase	Cholesterol
Lactate dehydrogenase	Lactate
Alcohol oxidase	Alcohol
Hexokinase	ATP
Galactose oxidase	Galactose
Penicillinase	Penicillin
Ascorbic acid oxidase	Ascorbic acid
L-Amino acid oxidase	L-Amino acids
Cephalosporinase	Cephalosporin
Monoamine oxidase	Monoamine

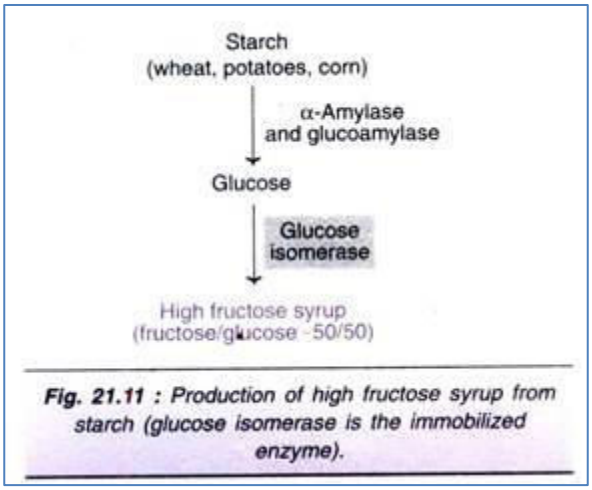


Fig. 21.11 : Production of high fructose syrup from starch (glucose isomerase is the immobilized enzyme).

Glucose Isomerase:

This is an intracellular enzyme produced by a number of microorganisms. The species of Arthrobacter, Bacillus and Streptomyces are the preferred sources. Being an intracellular enzyme, the isolation of glucose isomerase without loss of biological activity requires special and costly techniques. Many a times, whole cells or partly broken cells are immobilized and used.

Question Bank

Group A

1. Multiple Choice Question (MCQ)

i. Enzymes produced by microorganisms are

(a) Intracellular (b) extra cellular (c) both (a) and (b) (d) none of these.

ii. Which of the following enzymes is/are required in r DNA technology?

(a) Endonuclease ;(b) Polymerase ;(c) ligase ;(d) All of these

iii. Enzymes are

(a) Mixed growth associated product ; (b) Nongrowth associated product ;(c) Growth associated product;(d) none of these

iv. The organism generally used for α-amylase production by mold fermentation

(a) *Aspergilus niger*; (b) *Aspergillus parasiticus* ;(c) *Saccharomyces cerevisiae*;
(d) *Aspergillusoryzae*

v. Critical oxygen concentrations in cultures range about

(a) (2-5) %;(b) (5-25) %; (c) (30-50) %;(d) Greater than 50%

vi. Power number is a function of

(a) Impeller diameter ; (b) Speed of the impeller;(c) supplied power;(d) All of these

vii. OUR is dependent on

(a) K_L; (b) $K_L a$; (c) Impeller speed ;(d) All of these

viii. Vector used in r DNA technology is

(a) Plasmid ;(b) Cosmid ;(c) Fosmid;(d) All of these

ix. Enzyme is a

a) Carbohydrate molecule; (b) protein molecule; (c) Lipid molecule; (d) vitamin molecule in nature

x. Glucose isomerase converts

a) Sucrose to glucose and fructose ; (b) glucose to fructose;(c) lactose to glucose and galactose; (d) maltose to glucose

xi. Majority of enzymes used in food industry are

(a) Transfarases;(b) isomerases;(c) oxidoreductases;(d) none of these

xii. Enzymes produced by micro-organisms are

(a) Intracellular ;(b) extracellular ;(c) both (a) and (b);(d) none of these

xiii. Allosteric enzymes have

(a)only one substrate binding site; (b) more than one substrate binding site; (c) one or more substrate binding site; (d) no substrate binding site.

xiv. Holoenzyme contains

(a) only the protein part (b) only the non protein part (c) both the protein and non protein part (d) neither a protein part nor a non protein part

GROUP B

Short Question (SQ)

2. Name five enzymes with their specific one industrial application. 1+1+1+1+1=5

3. What are the advantages of using microbial source for enzyme production compared to animal and plant source? $2^1/_2+2^1/_2=5$

4. Describe intracellular and extracellular enzymes with examples. 5

5. Classify enzymes depending on their functions. 5

6. What is the role of rennin in cheese production? Give examples of cofactors. 4+1=5

7. Write a short note on Hindered settling. 5

8. Write the differences between inorganic catalyst and enzyme. 5

9. What is coenzyme? Compare between feedback inhibition and feedback repression. 1+4=5

10. What is rDNA technology? Briefly explain the role of different enzymes in rDNA technology? 1+4=5

11. What is chimeric DNA? What is the role of vector in r DNA technology? Give one example each of natural and artificial vectors. 1+2+2=5

12. Give some potential applications of r DNA technology5

13. Why microbial source is favorable for enzyme production in comparison to other sources? What should be the criteria of a microorganism selected for enzyme production? 2+3=5

14. Write down a flow diagram showing the major steps of separation and purification of intracellular enzymes. **5**

15. Write the advantages and disadvantages of whole cell immobilization. **2+3=5**

16. Write the sources and commercial uses of glucose oxidase. **5**

17. Temperature has a two-fold effect on enzyme activity-explain. **5**

18. What is doubling time of cell mass? Prove that doubling time (τ_d) = ln 2/ μ_{net}. If in a food material initial number of microorganism is 10 and the doubling time of the organism is 20 min then find out the number of organisms in the food material after 1 hr. **1+2+2=5**

19. Describe the action of three different amylases upon starch molecule. Mention the strain, media composition, control parameters and recovery process of commercial production of α-amylase. **2+3=5**

21. What is growth associated, non growth associated and mixed growth associated product formation model? Cite one example each. **3+2=5**

GROUP C

Long answer type questions (LQ)

22. Classify the different cell disruption methods. Describe the principles of any three cell disruption methods. 6+3+3+3=15

23. a) Derive the Ruth equation for constant pressure filtration.

b) The following data were obtained in a constant pressure filtration unit for filtration of a yeast suspension.

t **(min)**	**V** **(L filtrate)**
4	115
20	365
48	680
76	850
120	1130

Characteristics of the filter are as follows:

A = 0.28 m^2, C = 1920 kg/m^3, $\mu = 2.9 \times 10^{-3}$ kg/m-s, α = 4m/kg

Determine

i) Pressure drop across the filter.

ii) Filter medium resistance (r_m) **7+8=15**

24. Write the different types of enzymes used in bread industries. What is the role of lipoxygenage in bread production? Write the function of pectinase in fruit juice industry. 6+5+4=15

25. How the height of liquid in fermenter and the types of agitator affect the O_2 transfer rate. Describe the types of sparjers used in fermenter. Describe the sulfate method of determination of $K_{La.}$ 7+4+4=15

26. a) Explain the Dynamic method for determination of Volumetric Oxygen Transfer Coefficient (k_La) of a fermenter.

b) The following data were obtained for dissolve oxygen measurement in a glucose-gluconic acid fermentation system using a 5L fermenter.

Time (sec)	**D.O. (mg/L)**
10	3.5
15	3.5
25	3.5
30	3.5
35	3.5
40 (air off)	3.5
45	3.0
50	2.5
55	2.0
60	1.5
65 (air on)	1.0
68	1.4
73	1.85
80	2.25
88	2.55
95	2.75
103	3.00
111	3.25
117	3.30
122	3.40
130	3.45

Determine the values of

i) k_La

ii) rX

iii) C*

27. Draw a flow diagram of enzyme production by microorganisms, mentioning each steps. Which factors are considered at the time of selection of microorganisms for enzyme production? Write the advantages of submerged culture fermentation. 6+5+4=15

28. (a) A stirred tank reactor is to be scaled down from 10 m^3 to 0.1 m^3. The dimensions of the large tank are: Dt = 2m, Di = 0.5m, N = 100rpm
(a) Determine the dimensions of the small tank (Dt, Di, H) using geometric similarity.
(b) What would be the required rotational speed of the impeller in the small tank if the following criteria were used?
i) Constant P/V
ii) Constant tip speed.
iii) Constant impeller Reynolds Number
(b) What is aeration number and impeller's Reynolds number. **10+5=15**

29. Convert the Michaelis-Menten equation to Lineweaver- Burk formula. Determine the values of Vm and Km for the following set of data of an enzyme catalyzed reaction using Lineweaver-Burk formula

v (mmol / L-min)	**S (mol / L)**
0.083	0.010
0.143	0.020
0.188	0.030
0.222	0.040
0.250	0.050
0.330	0.100
0.408	0.290

3+12=15

30. Write the principle of any **three** enzyme purification method. **3x5=15**

31. What are the advantages and disadvantages of cell immobilization over enzyme immobilization? What are the criteria of a matrix to be selected for immobilization? Give **five** specific examples of industrial application of immobilized enzyme. 5+5+5=15

32. a)What are the criteria of an organism to be selected for industrial use?
b)What is enzyme Immobilization? What are the advantages and disadvantages of immobilization? What are the methods of enzyme immobilization? **4+ 1+5+5=15**

33. Write the different types of enzymes used in bread industries. What is the role of lipoxygenage?
in bread production. Write the function of pectinase in fruit juice industry. **6+5+4=15**

34. What is activation of enzyme? Write the effect of temperature and pH on enzyme activation. What is Q_{10}value? **2+12+1=15**

35. (a) Give the mathematical expression of Monod equation with significance of each term. What do you understand by growth limiting substrate? What is the limitation of this equation? **3+1+1=5**

(b) A strain of mold was grown in a batch culture on glucose and the following data were obtained

Time(hr)	Cell concentration(g/l)	Glucose concentration(g/l)
0	1.25	100
9	2.45	97
16	5.1	90.4
23	10.5	76.9
30	22	48.1
34	33	20.6
36	37.5	9.38
40	41	0.63

(i) Calculate the maximum net specific growth rate
(ii) Calculate the apparent growth yield
(iii) What maximum cell concentration could one expect if 150gm of glucose were used with the same size of the inoculum? **10**

36. Draw a neat sketch of a CSTF. Describe the function of sparger, impeller and baffle in a reactor vessel. What is power number? With diagram show the difference between Bubble column and Loop reactor. **5+4+1+5=15**