ENZYMES IN FOOD INDUSTRY

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BOOKS ARCADE

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CHAPTER 1

ROLE OF ENZYME IN THE FOOD SECTOR

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The basic building component of living systems, the cell is the structural or functional unit of life. Enzymes are biocatalysts with exceptional catalytic efficiency and substrate or reaction specificity that may be used efficiently by cells. Enzymes are ideal for biological processes due to their extraordinary catalytic activity and high level of substrate selectivity. They are essential for the metabolism of cells.

Enzymatic catalysis is directly responsible for every single chemical reaction that occurs in plants, microorganisms, and animals and causes it to occur at a measurable pace. The majority of biochemistry's history may be connected to enzyme research's history either directly or indirectly. Early 1800s study on the digestion of meat led to the first reports of catalysis in biological systems. The catalytic activity of stomach secretions, saliva's conversion of starch to sugar, as well as other plant extracts were all described in this study, .The main structure of polypeptides, secondary protein structures, and tertiary structure will all be covered in this session. It will be explained what an enzyme active site is.

Protein macromolecules make up enzymes. With the exception of catalytic RNA and DNA which are not proteins but rather considered enzymes.

1. They generally range in length from 100 to 500 amino acids and have a well-defined amino acid sequence.
2. Their three-dimensional structure is well recognized.

Catalysts are enzymes.

1. With a known mechanism, they serve as a catalyst for chemical or biological reactions.
2. They accelerate the reaction more quickly than it would have otherwise, often by a factor of 106 to 1014.
3. They have a narrow substrate preference.
4. By decreasing the activation energy, they increase response rate.
5. They are stereospecific, which means that just one product is produced by the reaction.

Primary Structure

Amino acids are the building blocks of enzymes, and they are connected in a linear chain by amide bonds. The main structure is this. Polypeptides or proteins are the names given to the

resultant amino acid chain. The DNA sequence of the relevant gene, as illustrated in Figure 1.1, encodes the precise order of amino acids in the protein.

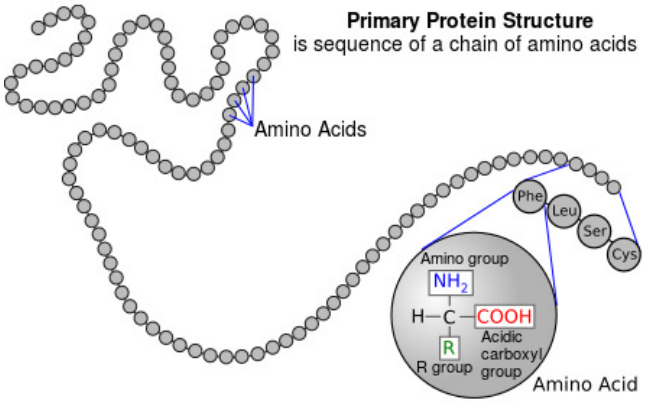

Figure 1.1: Illustrate the Primary Protein Structure.

Secondary Structure

Because each amino acid has an amino group and a carboxyl group , which may form a hydrogen bond with each other, the amino acids in the same chain can interact with one another. In order to create two secondary structures, the protein chain could fold up on itself in one of two different ways, as illustrated in Figure 1. 2. It can either wrap around to form a -helix or fold on top of itself to make a -sheet.

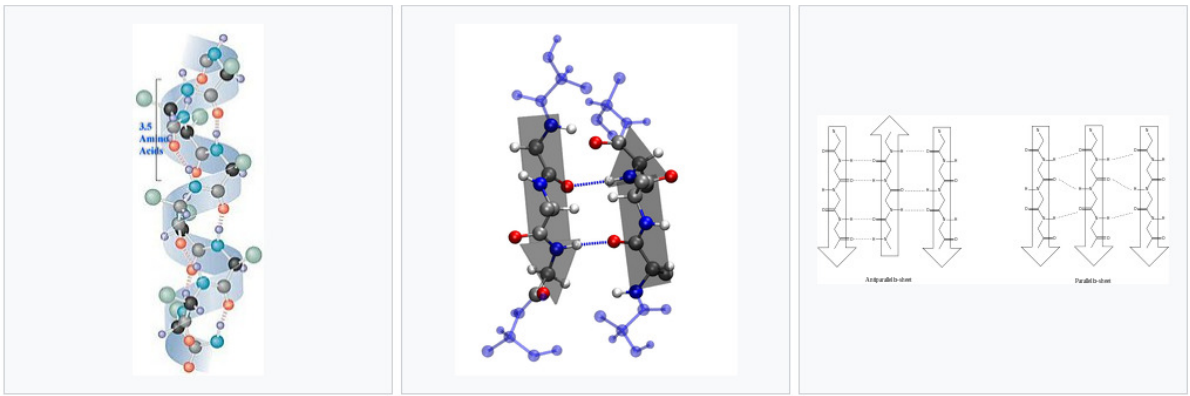

Figure 1.2: Illustrate the in the hydrogen bonds are shown by the dotted lines in the aforementioned photographs. Depending on the orientation of the protein chain, there are two different types of -sheets. An anti-parallel sheet is created when the direction changes with each fold, a parallel sheet is created when the direction stays the same.

Tertiary Structure

The protein can fold up further and acquire a three-dimensional structure as a result of the folding-up of the 2D linear chain in the secondary structure. Figure 1.3 depicts its tertiary structure in this manner.

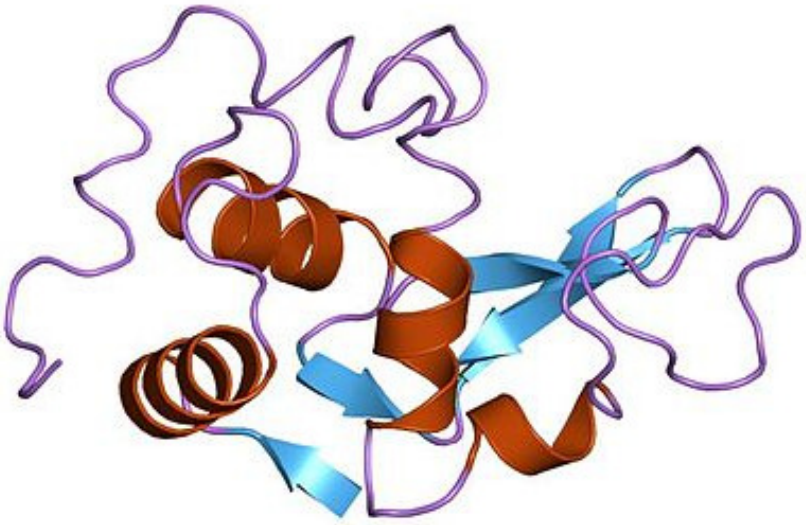

Figure 1.3: Illustrate the Lysozyme, the Blue Arrows Represent the Sheets, while the Red Helices are Helices.

Enzymes

For over a century, scientists have known that enzymes exist in nature. However, James B. Sumner isolated the first crystalline enzyme urease from the jack bean in 1926. Enzymes, also known as biocatalysts, are biological substances that initiate or speed up the rate of a metabolic process in a living organism while not being consumed in the process. Despite the fact that enzymes are produced within living cells, they can function actively in vitro, making them useful in industrial processes..Enzymes are complex protein molecules that are produced by living organisms to catalyse the biochemical reactions needed to sustain life. Enzymes are mostly proteins, but not all of them. RNA and antibodies can both function as catalysts known as ribozymes and abymes. According to the literature, enzymes catalyze over 5000 different biochemical reactions. Enzymes, like other chemical catalysts, are extremely effective at increasing the rate of biochemical processes that would otherwise proceed very slowly, or in some cases not at all (Figure 1.4).

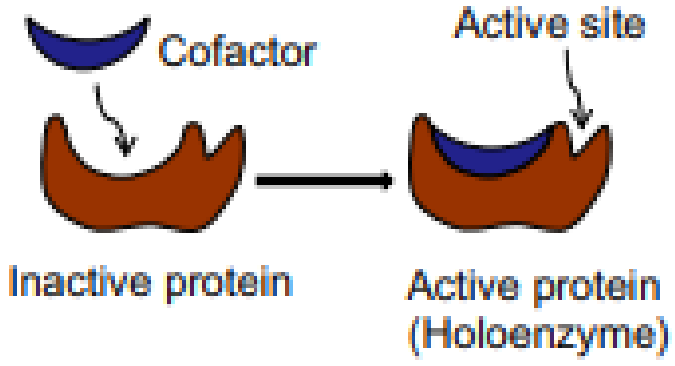

Figure 1.4 Represent the Holoenzyme.

In most cases, no. A common example is the breakdown of foods into their basic constituents, which primarily include proteins, carbohydrates, and fats. It is usually completed within 3-6 hours, depending on the type and quantity of food. However, in the absence of enzymes, this food breakdown would take more than 30 years. Enzymes are more specific in action and have higher catalytic properties than chemical catalysts. Enzymes can also be immobilised on inert material without losing activity, allowing for reuse and recycling.

Most, but not all, enzymes require a small atom to function as a catalyst. Cofactors or coenzymes are the names given to these molecules. Cofactors are non-proteinaceous chemical compounds that bind to an inactive protein part of an enzyme to increase the enzyme's biological activity required for its function. Holoenzyme is the active complex of an apoenzyme and a cofactor. Cofactors are also known as "helper molecules" because they aid in biochemical transformations. Cofactors are classified into two types: coenzymes and prosthetic groups.

Coenzymes are organic molecules that bind to enzymes and aid in their functions. They are a type of cofactor. Organic molecules are simply carbon-containing molecules. Vitamins are the source of many coenzymes. These molecules are frequently attached to an enzyme's active site and aid in the transport of a substrate or product. They can also shuttle chemical groups from one enzyme to another. Coenzymes bind loosely to the enzyme, but another class of cofactors does not. Prosthetic groups are indeed cofactors that frequently form covalent bonds with proteins or enzymes. One of the most important properties of enzymes is their specificity for the substrates or reactions they catalyse, which makes them useful as a research and industrial tool.

Enzyme Units

Typically, the amount of an enzyme in a specialised biochemical reaction is determined by measuring the rate of the reaction. An enzyme's activity is measured in enzyme units. The International Union of Biochemistry's Enzyme Commission recommends expressing it in International Units, the most widely used unit of enzyme activity. One IU is characterized by the quantity of enzyme required to catalyse the conversion of one micromole of substrate per minute under standard temperature, pH, and substrate concentration conditions. In addition, in order to adhere to SI units, the Panel on Biochemical.

Nomenclature recommended that reaction rates be expressed in moles per second and proposed katal, a new unit of enzyme activity. A katal is the amount of enzyme required to catalyse the conversion of one mole of substrate per second. Because the katal has such a large magnitude, it is rarely used in practise. The former IU is still more widely used, and its magnitude is adequate for measuring most enzyme preparations. The percentage of enzyme units per milligramme of protein is an enzyme's specific activity. As a result, the value of specific activity is expressed in units/mg, and it is an important indicator of enzyme purity.

Mechanism of Enzyme Action

In order for a reaction to occur, the chemical reactions.must expend some energy in order to cross the reaction's transition state and become products. This energy is referred to as activation energy, and it is defined as the smallest amount of energy required to activate all of the

molecules or atoms in 1mol of substance in order to achieve the transition state at the top of the energy barrier at a given temperature. It is assumed that proteases reduce the activation energy of enzymes catalysed in chemical reactions, increasing the rate of reaction by 106 to 1024-fold over nonenzymatic reactions. In other words, enzymes are thought to shorten the "path" of the reaction, requiring less energy to convert each molecule of substrate into products. As a result, the response is said to be faster in a given amount of time (Figure 1.5).

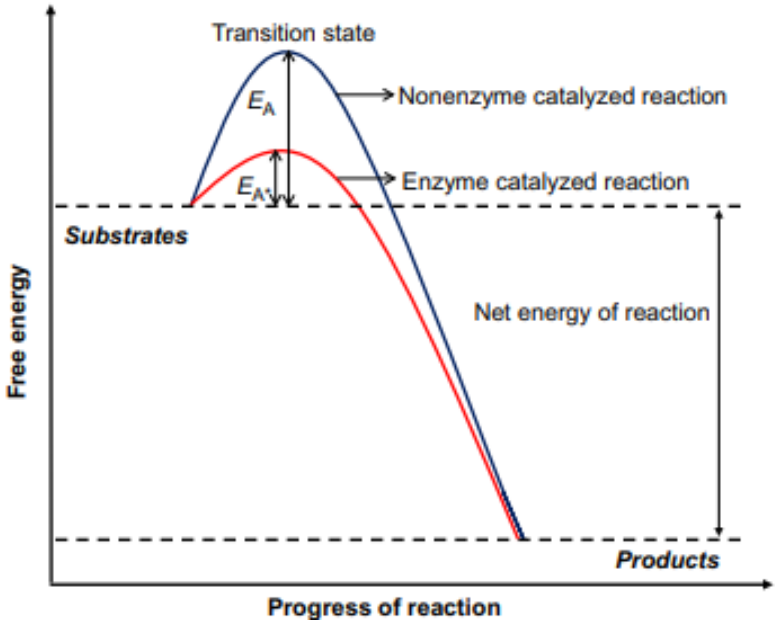

Figure 1. 5 represents the A schematic diagram comparing enzyme catalyzed reaction and no enzyme catalyzed reaction.

Svante Arrhenius, a Swedish chemist, proposed an explanation for an enzyme's catalytic action in 1888. He proposed that the enzyme and substrate combined to form an intermediate compound known as that of the enzyme-substrate complex. This complex was broken down into a product and an active enzyme. The overall enzyme-catalyzed reaction is represented as follows:

$$\underset{\text{Substrate}}{S} + \underset{\text{Enzyme}}{E} \rightarrow \underset{\text{ES complex}}{ES} \rightarrow \underset{\text{Product}}{P} + \underset{\text{Enzyme}}{E}$$

In general, enzyme-catalyzed reactions proceed as follows:

a) Noncovalent bonds connect the substrate molecule to the active site of the enzyme. The region of an enzyme that manages to combine with the substrate is known as the active site.

b) The enzyme and substrate combine to form an enzyme-substrate complex.

c) The substrate molecule is converted into a product by either atom rearrangement or substrate breakdown/joining with another molecule.

d) The breakdown of the ES complex results in the formation of a product, which is released from the active site of the enzyme.

e) The enzyme's nature remains unchanged and it can catalyse a new reaction.

Two proposed models explain the processes of enzyme action in general:

Lock and Key Design

Emil Fischer proposed this theory in 1894, claiming that both a substrate and an enzyme have specific geometric shapes that fit perfectly together. It specifies that an enzyme's active site has a distinct conformation.

The lock and key hypothesis states that this active site is complementary to the substrate structure and thus allows the two molecules to fit together. The rigidity of the enzyme's catalytic site was recommended by this model.

Model of Induced-Fit

Another scientist named Daniel Koshland postulated some changes to the previously explained lock and key hypothesis in 1958. He proposed that the essential functional groups on the free enzyme's active site are not in the best positions to promote catalysis.

Because enzymes are so adaptable, when a substrate molecule binds to it, the active site of the enzyme takes on a favourable geometrical shape, resulting in the transition state.

According to Koshland, the substrate causes a conformational change in the enzyme, which aligns the amino acid residues or other groups for substrate binding and catalysis. This is known as the induced-fit model of enzyme action.

Factors Affecting Enzyme Activity

Several factors influence enzyme activity or the rate of an enzyme-catalyzed reaction. Temperature, pH, substrate density, concentration, and the presence of any inhibitors or activators are among these factors.

Effect of Temperature

Temperature influences enzyme activity, or the rate at which an enzyme catalyses a reaction. All enzymes have a temperature range at when they're normally active. The temperature at which enzymes work best, or where the rate of reaction is fastest, is referred to as the optimum temperature.

This temperature varies depending on the enzyme. Because most enzymes are proteins, heat denatures them and causes them to become inactive when the temperature rises above a certain spot. Some enzymes become inactive (Figure 1.6).

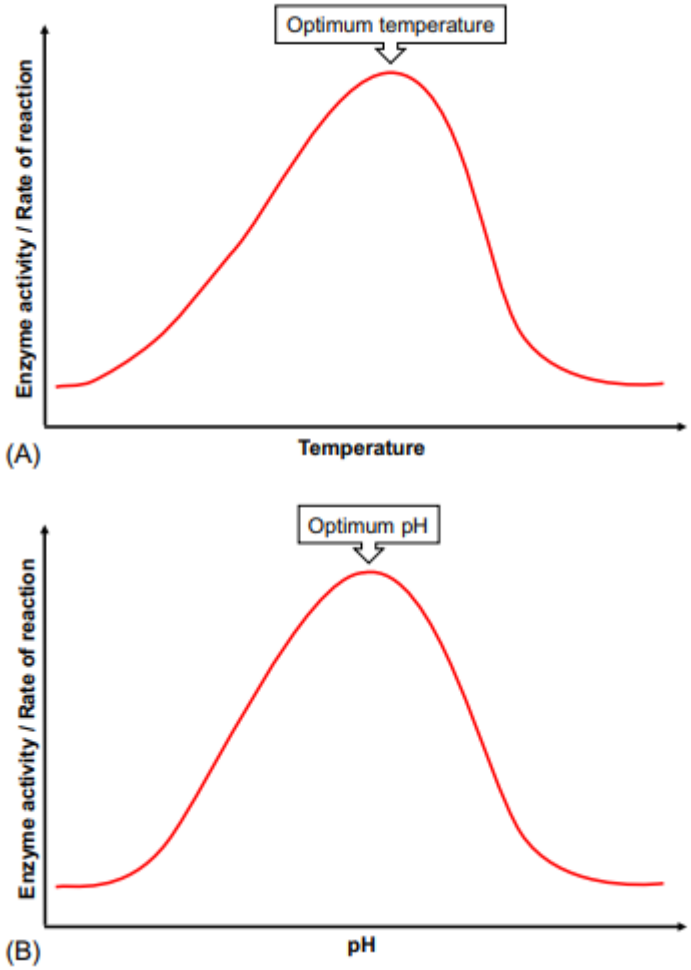

Figure 1.6 Represents the Effect of temperature on enzyme activity or enzyme catalyzed reaction Effect of pH on enzyme activity or enzyme catalyzed reaction.

When it is frozen. However, some enzymes perform exceptionally well at low temperatures, such as 4°C, and others perform exceptionally well at high temperatures, such as 95°C . Enzymes are inactive at low temperatures. However, over a limited temperature range, the rate of oxidase reactions roughly doubles with a 10°C increase in temperature.

Effect of pH

The pH, like temperature, influences enzyme activity or the rate of enzyme-catalyzed reactions. A solution's acidity or basicity is determined by its pH value, which ranges from 1 to 14. The pH of acidic solutions is 7, and the pH of basic.solutions is greater than 7. Deionized water has a pH of 7, indicating that it is a neutral solution. The majority of enzymes have a specific pH value at which their activity is at its peak. This pH is known as the optimum pH, and when it is exceeded or depleted, enzyme activity or the rate of enzyme catalysed reaction decreases. The optimal pH value for each enzyme varies. Small pH deviations from their optimal value do not permanently denature an enzyme.

Although extreme pH changes reduce enzyme activity and permanently denature the enzyme. Because of the differences in environmental conditions, enzymes present in different locations have varying pH values. Furthermore, some enzymes work best at acidic pH levels, known as acidophilic enzymes, while others work best at alkaline pH levels, known as alkaliphilic enzymes.

Effect of Substrate Concentration

The rate of an enzyme-catalyzed reaction is also affected by substrate concentration. When the substrate concentration is increased, the recorded velocity of the reaction forms an oblong hyperbola if other factors such as pH, temperature, and enzyme concentration are at optimal levels.

At first, the reaction velocity is nearly proportional to the substrate concentration. Furthermore, as the substrate concentration increases, the rate of reaction slows and eventually becomes constant. Increasing the substrate concentration causes the reaction rate to rise in the initial phase because more substrate molecules bind with enzyme molecules, resulting in more products formed. All of the enzyme molecules are saturated with substrate molecules at maximum velocity, so further increases in substrate concentration cannot result in an increased reaction rate.

Effect of Inhibitors

Enzyme inhibitors are molecules that socialise with enzymes in some way, either temporarily or permanently, to reduce the rate of an enzyme-catalyzed reaction or to prevent enzymes from working normally. Competitive, noncompetitive, and uncompetitive inhibitors are the most common types of inhibitors. Aside from these inhibitor types, there is also mixed inhibition.Competitive enzyme inhibitors have a similar shape to the substrate molecule and compete with it for the enzyme's active site. This keeps enzyme-substrate complexes from forming. As a result, fewer molecules can bind to the enzymes, slowing down the reaction rate. The level of inhibition is determined by the concentration of substrate but also inhibitor. This is a process that can be reversed. In the case of inhibiting the activity, Km increases while Vmax remains unchanged. Noncompetitive enzyme inhibitors bind to an allosteric site, which is different from the active site of the enzyme.

This binding deforms the structure of the enzyme, preventing it from forming the ES complex at its normal rate and preventing the formation of isoenzymes, resulting in fewer product formations. Noncompetitive inhibitors are unaffected by substrate concentration because they do not compete with substrate molecules. In the case of noncompetitive inhibition, Vmax is reduced but Km remains unchanged. Uncompetitive inhibitors can only bind to the ES complex and not to the free enzyme. The resulting ES complex is inactive enzymatically.This type of inhibition is uncommon, but it can happen in multimeric enzymes. Some enzyme inhibitors covalently bind to the enzyme's active site and inhibit its total activity, resulting in enzyme poison. This type of inhibition is permanent. Some enzyme inhibitors can be used as a drug or as a metabolic poison to treat a specific disease.

Sources of Enzymes

Enzymes are found in all living things. Commercial enzymes, on the other hand, are primarily derived from three primary sources: animals, plants, and microorganisms. Microorganisms are preferred as sources of industrial enzymes over other sources for the following reasons:low production cost,more predictable and controllable enzyme contents of microbes,easy availability of raw materials with consistent composition for their cultivation, but also.plant and animal tissues contain more possibly harmful materials than microbes. Microbes are isolated from their natural environments and used to produce the desired enzyme by optimising their growth conditions.

Fermentation is the name given to this process. The following are some important food enzymes and their sources: Catalase, lipase, and rennet are examples of animal enzymes. Plant enzymes include actinidin, -amylase, -amylase, -glucanase, ficin, lipoxygenase, and papain. Bacterial enzymes: -amylase, -amylase, glucose enzyme that converts, protease, pullulanase; Fungi enzymes include: -amylase, catalase, dextranase, glucose oxidase, lactase, lipase, pectinase, protease, and raffinase; yeast enzymes include: invertase, lactase, lipase, pectinase, protease, and raffinase.

Enzymes in Food Technology

Enzymes have been used in the production of various foods since ancient times. One of the most common examples is the use of an industrial enzyme in the production of beverages such as whisky, beer, and wine. The food industry now employs a variety of screening technologies to discover new food enzymes, but it has become difficult for food scientists and biochemists to deliver new food enzymes for commercial applications. However, biotechnology has emerged as a cutting-edge tool for the food industry. This technology creates new products, improves nutritional value, lowers production costs, improves food processing, and addresses waste, food safety, and packaging issues. This will also play a significant role in future food manufacturing and assembly industries.

Enzymes or enzyme-catalyzed reactions are now used in almost all commercial foods or their ingredients. Alcoholic beverages, syrups, sweeteners, chocolates, infant foods, bakery products, yoghurt and dairy products, egg products, fruit juice, soft drinks, candy, flavour development, meat tenderization, and so on are examples of such foods/processes.Enzymes have numerous benefits in food production and processing. The most important aspect is that enzymes are used to replace traditional chemical-based technology.

This reduces processing energy consumption, produces biodegradable products, and has a lower environmental impact. Furthermore, enzyme-catalyzed processes generate fewer waste products.than chemical catalysts due to the specific action of enzymes. Furthermore, enzymes can catalyse reactions under very mild conditions, avoiding the destruction of valuable food elements and food components. Although plants, animals, and microbes produce the majority of food enzymes, enzymes produced by microbial sources are now more advantageous than those produced by plants and animals.

Cold-Active Enzymes In The Food Industry

Cold-active enzymes have a wide range of applications in the food industry and biotechnology. Important applications include the dairy, juice, meat, and baking industries. Cold active -galactosidase, for example, is used in the dairy industry to reduce the amount of lactose in milk. Lactose, a disaccharide sugar, causes severe intolerances in the majority of the world's population. Pectinases are used in the fruit juice industry to reduce viscosity and refine the final product during the juice extraction process.

Cold-active proteases are used for the meat processing industry to tenderise meat. Some enzymes, such as proteases, amylases, and xylanases, can help reduce dough fermentation time while also retaining aromas and moisture levels in baked goods. Other cold enzymes may also be used as mesophilic and thermophilic enzyme substitutes in the brewing and wine industries, cheese manufacturing, and animal feed, and so on. Psychrophilic enzymes are commonly used in food biotechnology for meat tenderization, food processing, flavouring, baking, brewing, cheese production, and animal feed. According to the literature, coldactive enzymes have several advantages over mesophilic/thermophilic enzymes. The majority of cold-active enzymes are distinguished by their high catalytic efficiency at low to moderate temperatures, where homologous mesophilic enzymes are inactive.

The use of psychrophilic enzymes can be suitable not only because of their high specific activity, which reduces the amount of enzyme required, but also because they are easily inactivated. Cold-active enzymes have three important characteristics that increase their potential in biotechnology:.they are cost effective because fewer enzymes are required to meet the activation energy requirement;they are proficient without additional thermal aid; anddue to thermal lability, their selective inactivation can always be achieved with less heat input. As a result, we can conclude that cold-active enzymes have a lot more to offer in terms of food biotechnology. Even though cold-active enzymes have high specific activity but a short half-life, there is a significant disadvantage in commercialising these enzymes. To meet commercial expectations for cold-active enzymes, various molecular strategies such as protein engineering, r-DNA technology, and the metagenomic approach could be developed to achieve qualitative and quantitative improvements and to develop radically novel cold-active enzymes. Genetically improved microbes suitable for the production of specific cold-active enzymes would be useful in the food industry.

CHAPTER 2

MICROBIAL ENZYME IN FOOD BIOTECHNOLOGY

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The use of enzymes and microorganisms in food processing is a well-known approach. For centuries, enzymes and microorganisms have been utilized in the brewing of beer, bread baking, and the production of cheese and wine. Food biotechnology provides numerous methods for improving the processing of raw materials in order to convert them into superior dietary value food products. Enzyme technology improves food quality in a variety of ways, including increased functionality, nutritional value, and flavour and texture. Animal, plant, and microorganism sources of enzyme production exist, with microorganisms.being the best and most suitable sources of commercial enzyme production.Enzymes such as -amylase, galactosidase, lipase, pectinase, chymosin, and protease are used in food processing industries around the world. The -amylase enzyme converts starch into dextrins and produces corn syrup, which is used to improve the sweetness of various food products. Using barley and other cereal grains, the brewing process converts complex carbohydrates into simple carbohydrates. Glucoamylase.convert dextrins into glucose in the form of corn syrup during the production of high-quality light beer, converting residual dextrins into fermentable sugars. Lipases are fat-hydrolytic enzymes that improve flavour, speed up cheese ripening, and produce higher-quality special fat products.

Pectinase is a hydrolytic enzyme used in fruit juice extraction, clarification, and filtration. Chymosin enzymes aid in the breakdown of kappa-caseins during the milk curdling process. Enzymes from bacterial and fungal proteases are used to make fish meals, meat tinctures, texturized proteins, and meat extenders. Lactase enzymes convert lactose found in whey and milk products into polyactide. To stop maillard reactions, glucose oxidases convert glucose to gluconic acid. Acetolactate decarboxylase converts acetolactate to acetoin in order to shorten the maturation time in wine production. Cellulase is a polysaccharide-solubilizing enzyme that is not found in starches.

Sources of Enzymes

Originally extracted from the stomachs of calves, lambs, and baby goats, enzymes are now produced by microorganisms such as bacteria, microorganisms, yeast, and actinomycetes. Microorganism-derived enzymes outperform those derived from animals and plants. Microorganisms can indeed be genetically modified to improve commercial scale production.Enzymes can hydrolyze complex molecules into simple monomer units, similar to how carbohydrates can be broken down into simple sugars, which are natural substances involved in a variety of biochemical processes. For catalysing the reaction to convert a reactant into a product, each enzyme is substrate, pH, and temperature specific.

History of enzymes

Enzymes were once known as 'biocatalysts,' as they aided in the acceleration of biological or biochemical reactions. The term "enzyme" was coined in 1877 by Wilhelm Friedrich Kuhne, Professor of the Department of Physiology at the University of Heidelberg, in a paper to the Heidelberger Natur-Historischen und Medizinischen Verein, who proposed that such unorganised ferments be referred to as enzymes. It was derived from the Greek term 'v', which means 'in leaven' or 'in years

Though enzymes have been used by humans for centuries, the term "enzyme" was not coined until the 18th century. There were many ancient uses of enzymes, such as barley malt for starch conversion in brewing or calf stomach as a catalyst in the manufacture of cheese, before the nature and function of enzymes were understood. Later, many scientists reported on enzymes in various forms. For example, in around 1783, Spallanzani demonstrated that gastric juice secreted by cells could digest meat in vitro, and whose active substance was named pepsin by scientists. The first enzyme discovered was 'diastase' in 1833 by a French scientist, Payen, who discovered it catalyses the breakdown of starch into glucose in malt. In 1926, James B. Sumner of Cornell University isolated the first pure enzyme, known as urease. He was awarded the Nobel Prize in 1947 for isolating and crystallising the jack bean enzyme urease. John H. Northrop and Wendell M. Stanley of the Rockefeller Institute for Medical Research were also awarded the Nobel Prize in 1947 for their discovery of a complex procedure for isolating pepsin.

Microbial enzymes

Microbes, like all living cells, produce enzymes that are hydrolyzing, oxidising, reducing, or metabolic in nature, but the quantity of enzyme produced varies between species and strains. As a result, for commercial production of specific enzymes, a strain with the highest enzyme activity must still be chosen. Enzymes derived from microbial sources are more advantageous than those derived from plant and animal sources due to lower production costs, large-scale production, greater scope for gene engineering, rapid culture development, less material use, environmental friendliness, and a wide range of physical and chemical properties; thus, they are preferred in a variety of industrial applications. The use of microbial enzymes has largely contributed to significant advances in the food industry. Enzymes are increasingly being used in food processing due to a growing interest in clean label foods and environmental concerns.

Microbial enzymes and their uses since centuries

Microbial enzymes have been used for food applications since at least 6000 B.C, when neolithic people cultured fermented grapes to make wine and Babylonians used microbial yeast to make beer. Over time, mankind's understanding of the use of microbial enzymes grew, allowing the production of cheese, yoghurt, vinegar, and other foods. Rennet is a natural enzyme mixture derived from the stomachs of calves or other domesticated that has been used successfully in cheesemaking for centuries. Rennet was discovered by the Egyptians between 4000 and 5000 years ago, according to historical records. They were storing liquids in the dried intestines of animals, particularly the stomachs. The rennet enzyme produced by these stomachs caused milk to curdle, allowing it to be preserved. The art of making cheese has evolved over time, and

natural rennet has always been inextricably linked to cheese. Rennet contains a protease enzyme, which coagulates milk and causes it to separate in to other solid.and liquid components.

Sir Alexander Fleming extracted the wonder drug penicillin from mould in 1928, and large-scale production of penicillin began around 1940. Following that, the era of microbiology blossomed as a science, and our understanding of fermentation and its applications grew. As a result, the mid-nineteenth century saw the proper understanding and application of these microbial enzymes in food applications. One important reason was the European industrial revolution, which resulted in large-scale migration of people to cities, causing food scarcity and prompting the discovery of methods for bulk food preparation and commercialization. However, the biotechnology revolution did not begin until after World War II, giving rise to modern industrial biotechnology or a fi llip to microbial enzyme technology in food application.

Enzyme Production in Bioreactor

The development of bioreactors, which serve as the focal point of interaction between life scientists and process engineers, is critical to the continued success of biotechnology. As a result, bioreactors are indispensable tools for critical biotechnological processes such as commercial-scale enzyme production and other high-demand bio-products.

Extremophiles as a Potential Resource for Enzymes

Extremozymes have a wide range of applications in agriculture, chemistry, and pharmaceuticals, with significant economic potential. Extremophile enzymes have unique properties that make them valuable resources in biotechnology. The most attention has been paid to thermophilic extremophiles. Hyperthermophiles.and thermophiles.aid in the production of thermostable proteases, lipases, and polymer-degrading enzymes such as cellulases, chitinases, and amylases, which have found use in industrial applications. Many enzymes are known to be produced by thermophilics; for example, organism's amylase and glycosidases are used in the production of glucose and fructose as sweeteners, starch processors, scarifying enzymes, and so on.Nowadays, genetic engineering is a very useful tool for increasing enzyme production because it is more cost-effective, as well as for obtaining enzymes that are better adapted to the conditions used in modern food manufacturing processes. The former can also be attained by screening microorganisms from various environments, but this is a time-consuming process with no guarantee of success. They can also be expressed on fi lamentous fungi and yeasts. For the enhanced production of enzymes, genetic engineering technologies such as recombinant DNA technology, embryogenic fusion, and mutation are commonly used.

Production Processes of Enzymes

The screening of potential strains of bacteria and the fermentation process are the two main components of industrial enzyme production. Another important aspect of commercial enzyme production is the composition of the production media; suitable media contains byproducts, nitrogen compounds, and micronutrients that aid in the growth of microorganisms during the fermentation process. Once fermentation is complete, the downstream process, such as enzyme recovery, purification, and product formulation, can begin within a suitable carrier.

Protoplast fusion

The discovery of the parasexual cycle by Pontecorvo and Roper.has proven useful in biotechnology and genetic engineering for improving industrially important fungi. The use of this parasexual cycle in industry Fungi production was highly significant because most fungi do not have a sexual cycle. This is widely used in genetic engineering to improve enzyme production.It entails the joining of two genetically distinct protoplasts from different somatic tissues. Three Trichoderma species were used for inter-generic protoplast fusion for citric acid production: T. reesei, T. harzianum, and T. viride strain.The glycoamylase activity of Aspergillus Niger recombinants produced by protoplast fusion was 2.5 times that of the parental strain.The application of protoplast fusion aided in the development of a promising technique for obtaining strains with increased laccase production.A batch process that is fed, and.a continuous process. All media components are added at the start of fermentation in the batch process. Fed batch fermentation is similar to batch fermentation in many ways.

However, during the fermentation process, the production strain is fed an additional nutrient medium. Continuous fermentation is a steady state achieved by supplying fresh medium while harvesting from the fermenter at the same time. Fermentation can take place in one of two ways: solid-state fermentation or submerged fermentation. Bacterial enzyme production is almost entirely accomplished through submerged fermentation because bacterial cell growth and enzy me secretion are more appropriate in a submerged environment.

When compared to solid-state fermentation. Submerged fermentation involves inoculating sterilised production media with bacterial strains and maintaining proper fermentation parameters such as aeration, agitation, dissolved oxygen, rotation, temperature, and pH for 48-72 hours, depending on the bacterial strain. Solid-state fermentation processes, on the other hand, are ideal for fungal strains to produce valuable industrial enzymes for a variety of food processing techniques. For the production of enzymes, fungal filament prefers surface fermentation, also known as solid-state fermentation, while others use full metabolite fermentation. At a proper temperature, humidity, and moisture of the fermentation system, sterilised solid substrate, such as wheat bran, rice bran, and many other grains, supports the growth of fungal cell mass. After specific periods of fermentation, the products are harvested and keep on going downstream processing, such as cell debris filtration, purification, and enzymatic product formulation. In general, intracellular enzyme downstream processing is more complex than extracellular enzyme processing. Purification of enzymes is a very expensive step in filtration; commercially, chromatography is primarily used in the purification of enzymatic proteins. The main issue with the formulation of purified enzymes with a suitable carrier is to ensure enzymatic activity and stability, as well as easy release of the enzyme at the site of application. Otherwise, the enzymatic product's efficiency is reduced.

Enzymes in the Beverage Industry

Because of rising consumer demand around the world, there is a growing interest in the food and beverage industries. The beverage industry is one of the largest food processing industries. The food and beverage processing industry is divided into two categories: alcoholic and nonalcoholic

beverages. Nonalcoholic drinks include fruit juice and soft drinks, whereas alcoholic drinks include beer, wine, and whisky. Coffee and tea are also options.

Tea Carbonated soft drinksare the most commonly consumed, followed by bottled water coffee, and beer Food, particularly fruits and vegetables, contain polysaccharides like pectin and starch, which cause fouling during the filtration process. The presence of cell debris and small insoluble pectin species is known to be responsible for the immediate turbidity in freshly pressed fruit juices, whereas haze formation may be caused by prior polymerization or condensation, which leads to the formation of polymeric complexes between polysaccharides, sugars, metal ions, and proteins. The most common cause of haze formation in beer, wine, and clear fruit juices is protein-polyphenol interactions. Fruit juices for commercial use are refined with chemical agents such as bentonite and gelatin.

However, using enzymes instead of chemicals is a more cost-effective option that improves yields by lowering carbon footprint, energy consumption, and pollution Enzymes are used as food additives and processing aids in food. Most food enzymes are used as help benefit, while a few, resulting in the formation of new,1-4 and,1-6 glycosidic bonds. The starch industry also uses this property to produce branched exopolysaccharides, also known as isomaltooligosaccharides, from maltose or glucose syrups. However, over the last decade, there has been an increase in demand for new products with health benefits, particularly those related to the controlled release of glucose from starch polymers into the bloodstream. Because of this, -glucanotransferases have gained prominence, resulting in the development of new commercial products such as cycloamylose, cyclic cluster dextrin, zwitterionic, thermoreversible starch, resistant starch, and highly branched structure.

-Glucanotransferases act on amylose, amylopectin, maltodextrins, and glycogen as substrates. These enzymes cleave an,1-4 glycosidic bond of the donor molecule and transfer part of the donor to a glycosyl acceptor by forming a new,1-4.or,1-6.glycosidic bond while retaining the anomeric center's -configuration. Due to the fact that -glucanotransferases use polymeric substrates, the hydroxyl acceptor can also be located downstream on the glucose-enzyme intermediate, resulting in intramolecular transglycosylation and the formation of cyclic products.

Catalytic action of cyclodextrin glucan transferase

Cyclodextrin glucanotransferase, also known as cyclomaltodextrin glucanotransferase or cyclomaltodextrin glycosyltransferase, is an extracellular enzyme that catalyses the cleavage of starch into cyclic, non-reducing, 1-4 linked cyclomaltodextrins, also known as cyclod. Over the last few years, a number of reviews on the production, properties, and applications of cyclodextrins have been published. CGTases are primarily derived from bacteria. The first CGTase to die was that from Bacillus macerans, which was initially known as macerans amylase, and was later highly purified and crystallised. This enzyme catalyses the intramolecular transfer of 1-4 glycosidic bonds, resulting in the formation of cyclic maltodextrins. Its action was described as exo, with the ability to bind six, seven, or eight glucose molecules at the non-reducing end of a starch polymer chain, resulting in cyclic dextrins containing initially six , seven , and eight.glucose units.

Nakamura *et al.* completely elucidated the proposed catalytic mechanism. The non-reducing end of a flour chain enters the active-site pocket first. Following binding of the oligosaccharide chain, the enzyme cleaves the,1-4 glycosidic bond and transfers the chain to the C4 oxygen of the non-reducing end glucose unit to form a new,1-4 glycosidic bond, resulting in the formation of cyclic maltodextrin. The cyclodextrin then exits the active site, letting the enzyme to repeat the process. Higher cyclodextrins, known ascyclodextrins, have also been reported to be formed from 9 to 13 glucose units. -Cyclodextrins, like -, -, and -cyclodextrins, were discovered to be unbranched. The branching degree, on the other hand, increases with molecular size, reaching nearly 100% in the case of cyclodextrins both reported the creation of larger cyclodextrins with DP values ranging from 6 to 60. These cyclodextrins, however, are converted into smaller excipients.as the reaction progresses.

Applications of cyclodextrin glucanotransferase

In the industrial production of additives, starch is first liquefied by a heatstable -amylase before being cyclized and disproportionated by CGTase. A significant disadvantage of large-scale cyclodextrin production is that all enzymes used today produce a mixture of cyclodextrins. Nonetheless, specific cyclodextrins, such as -cyclodextrins, are sometimes required for the complexation of guest molecules of specific sizes or for their specific nutritional properties. Cyclodextrins, for example, is marketed as a non-digestible, fully fermentable dietary fibre that is used in carbonated and noncarbonated transparent soft drinks, dairy products, baked goods, and cereals.The glucose residues of cyclodextrin rings are arranged so that the inside is hydrophobic, creating an apolar cavity, and the outside is hydrophilic.

As a result, cyclodextrins can form inclusion complexes with a wide range of hydrophobic guest molecules. This has sparked considerable interest, leading to the development of numerous cyclodextrin applications. Inclusion complex formation causes changes in the physical and chemical characteristics of the guest molecules, such as the stabilisation of light- or oxygen-sensitive compounds, the stabilisation of volatile compounds, the improvement of solubility, the extraction of cholesterol from foods, the improvement of smell or taste, or the modification of liquid compounds to powders 004). Furthermore, the ability to catalyse glycosylation reactions is being used in the production of stevioside, an intense sweetener isolated from the leaves of the herb Stevia rebaudania. CGTase treatment increases the solubility and decreases the bitterness of stevioside.

Catalytic action of 4-α-glucanotransferase

4--Glucanotransferase.transfers amylose fragments or parts of amylopectin side chains to the non-reducing ends of amylopectin, resulting in the formation of new,1-4 glycosidic linkages by breaking a -1,4 bond between two glucose units and then forming a new -1,4 bond. In terms of the enzymatic reaction, amylomaltase is very similar to cyclodextrin glycosyltransferase.The primary distinction is that amylomaltase continues to perform a transglycosylation reaction that yields a linear product, whereas cyclodextrin glycosyltransferase yields a cyclic product. Amylomaltases have been discovered in Eukarya as well as bacteria and Archaea, where they are involved in the utilisation of maltose or glycogen degradation. Despite the presence of short side

chains, the presence of long side chains with a length of 35 glucose residues or longer, combined with the starches' relatively high molecular weight, causes these starches to form a white opaque network or gel. However, unlike regular starch, this gel is thermoreversible, which means that the amylomaltase-treated starch can go through cycles of heating and cooling in which it dissolves when heated and gels when cooled, as opposed to regular starches, which cannot be dissolved in water after being retrograded.

The gel strength and paste flow behaviour of amylomaltase-treated starches are related to the incubation conditions and enzyme to starch ratio, whereas the melting temperature is independent of the reaction conditions and enzyme dosage. Amylomaltase-treated starch can be used in gum confectionery as well as as a fat replacer in mayonnaises and low fat products to create a creamy texture. Except for amylomaltase-treated starch, which forms a turbid gel, the thermoreversible characteristics of these starches also are found in gelatin.

Catalytic action of branching enzymes

Glycan branching enzymes play a role in the biosynthesis of amylopectin or glycogen in plants. These enzymes first break, 1-4 glycosidic bonds and then form new, 1-6 branches within linear, 1-4 segments, producing products devoid of long, 1-4 chains. BEs are found in plant and animal tissues, as well as microorganisms. Glycogen branching enzyme.is in charge of forming the,1-6 linkages in the glycogen molecule, whereas starch branching enzyme.is a plant analogue of glycogen branching enzyme that introduces -1,6-branches into amylose and amylopectin.

However, there are significant differences in the actions of GBE and SBE, as evidenced by the degree of branching between glycogen and amylopectin. The degree of branching in glycogen is 8-9% and 3.5% in amylopectin, with an average chain length of 10-12 glucose residues for glycogen and 20-23 glucose contaminants for amylopectin.These differences are thought to be primarily due to differences in the size of transferred chains between GBE and SBE. Furthermore, some SBEs have been reported to act solely on amylose whereas others have been reported to act on both amylopectin and amylose.

BE are derived from microorganisms, particularly bacteria. One of the most studied BEs is Bacillus stearothermophilus, which has an optimum temperature of 55^{o}C. Streptococcus mutans, Deinococcus geothermalis, and Deinococcus radiodurans have all produced enzymes with comparable thermal stability.

However, food industries interested in these enzymes want them to be more stable. Other microorganisms that produce these enzymes have also been studied in this manner. Shinohara *et al.* reported the first real BE from Rhodothermus obamensis, which is optimally active at 65^{o}C and stable up to 80^{o}Ca temperature at which gelatinized starch can be processed. Later, Gruyer *et al.* discovered a branching enzyme active at 80^{o}C in the hyperthermophilic archaeon Thermococcus hydrothermalis. More recently, the Aquifex aeolicus glucan branching enzyme was overexpressed in E. coli, and it was discovered that this enzyme is active at 80^{o}C. So far, this enzyme, along with the one from Rhodothermus obamensis, is the most commercially exploited BE.

Lipase

The use of enzymes in industrial processes is gaining popularity due to their availability and advantages over chemical catalysts, such as higher specificity, lower energy consumption, and increased reaction speed. Furthermore, enzymatic catalysis allows for improved product quality, lower production costs, and a lower environmental impact. As a result, the protein industry has grown steadily. The global market for industrial enzymes was estimated to be $3.3 billion in 2010 and is expected to grow to $8.0 billion by 2015. Lipase is currently the third most commercialised enzyme.

Lipases are enzymes that catalyse the total or partial hydrolysis of fats and oils, releasing free fatty acids, diacylglycerols, monoacylglycerols, and glycerol. These enzymes are distinct from esterases, which only act on moisture carboxylic ester molecules. Lipases can also catalyse synthesis reactions such as esterification, transesterification, aminolysis, and lactonization. Lipases are widely used in a variety of industrial sectors due to their unique properties, including food, pharmaceutics, biofuels, oleochemical, textile, agro-chemical, paper manufacturing, cosmetics, and many others. Lipases can be used in the food industry as flavour modifiers by synthesis of short chain fatty acid esters and alcohols, as well as to obtain products with higher nutritional value by modifying the triacylglycerol structure for inter- or transesterification. Lipases are potential emulsifier substitutes in the bakery. These metabolites are used in the wine industry to produce distinctive wine esters. Furthermore, hydrolysates can be used in a variety of processes, including the synthesis of structural lipids, low calorie lipids, and milk fat, as well as the ripening of cheese.

Microbial Sources of Lipases

Lipases are found in all living things and are produced by plants, animals, and microorganisms. However, microbial lipases, whether native or recombinant, are commonly used in a variety of biotechnological applications.Nature provides a diverse range of microbial resources. Microorganisms have remarkable adaptability, even in hostile environments such as the Dead Sea, Antarctica, alkaline lakes, hot springs, volcanic vents, and contaminated soils, which offer remarkable potential for the production of enzymes with specific properties. In this sense, the proportion of enzymes utilized by the food industry is constantly increasing, with prospects for further growth in the coming years due to demand for new applications in dairy and baking, among other fields. According to research, marine microorganisms have the ability to produce active compounds such as proteins and enzymes.

Boost production. A bacterial lipase allele from *Bacillus subtilis* was expressed in *Saccharomyces cerevisiae*, resulting in a significant increase in lipase production. According to the authors, this consists of a mixture has a wide range of applications in the bread industry. Alternative substrates, such as industrial waste or by-products, are another option for lowering lipase production costs. Marques and colleagues demonstrated that dairy effluent is a useful medium for *Trichoderma atroviride* 676 lipase production, lowering fermentation costs and contributing to the reduction of environmental issues brought on by the dairy industry. By-products of residual chicken fat and soybean oil were also used in the production of lipase by a

strain of *Pseudomonas sp.* Ramani and colleagues also demonstrated lipase production by *Pseudomonas gessardii* on beef tallow as a substrate.

Changes in texture, microstructure, and free fatty acid content of lighvan cheese were also reported during accelerated ripening with lipase from *Rhizomucor miehei*. The addition of lipase resulted in the accumulation of long, medium, and short-chain fatty acids, an increase in cheese hardness, and a decrease in the average diameter of the fat globules trapped in the clot network. After 90 days of maturation, the fat globules vanish completely and the protein matrix expands, filling the empty spaces left by the fermentation process.Lipases from *Rhizomucor miehei* are commercially available in soluble and immobilised forms and can be used in dairy processing. The genome of *R. miehei* CAU432 contains 40 glycerol-ester hydrolase genes, including true lipases (triacylglycerol lipases). Furthermore, the fungus contains 29 genes that are true phospholipases (lisofosfolipases) and 24 genes that are thiolester and sulfuricester hydrolases.

Phospholipases are an important group of enzymes used in dairy processing to increase fat retention in rennet curd. Libaek and colleagues investigated the effect of phospholipase A1 on the production and functional properties of mozzarella cheese and discovered that phospholipase enzymatic treatment increased fat retention in cheese due to an increase in lysophospholipids in the cheese curd.Concerning the microbial lipase used in dairy processing, Rhizopus oryzae is a major zygomycetes fungi that has been approved by the FDA as safe. When enzyme-modified cheese (EMC) is incubated at high temperatures in the presence of lipases or proteases, it produces a concentrated flavour with a 10 to 30 time's higher intensity than regular cheese. The flavour of cheese is very complex and varies depending on the type of cheese. The flavour and intensity are determined by the enzymes, starter microorganisms, fermentation, and emulsifiers used in the manufacturing process. Indeed, knowing the activity and specificity of each enzyme is required to control production.

Specific methylketones produced by Penicillum in lipidic medium contribute to the distinctive flavour of blue cheese. Cao and colleagues investigated the production of methylketones in blue cheese by lipase from Penicillum Roquefort, employing gas chromatography and sensory methods to evaluate the volatiles products. Lipase-treated blue cheese contained three times the amount of methylketone as untreated blue cheese. P. Roquefort's production of methylketones has been used in the cheese industry to provide the distinctive flavour of blue cheese. Methylketones appear to be produced by a futile cycle involving incomplete -oxidation of fatty acids, with the formation of medium-chain 3-oxoacyl-CoA intermediates that are converted by thioesterases, releasing coenzyme A and the 3-oxoacids that are decarboxylated to methylketones. The peroxisomal -oxidation pathway may be involved in the process.

CHAPTER 3

KINETIC AND PHYSICOCHEMICAL CHARACTERISTICS OF MICROBIALLIPASES

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Knowledge of the kinetic and physicochemical properties of microbial lipases is critical in the selection and application of lipases in food production. Furthermore, these characteristics are critical in distinguishing lipases from esterases. Both enzymes are hydrolases that catalyse the hydrolysis of carboxylic esters and are used in food production. Interfacial activation and the presence of an amphiphilic domain covering the active site were initially used to distinguish lipases from esterases. The increased hydrolytic activity observed when lipase is at the water/lipid interface is known as interfacial activation. This phenomenon is caused by an enzyme conformational change when the lid's oligopeptidic sequence is at the water/lipid interface. The movement of the lid allows lipid to reach the active site of lipase.

However, since the 1990s, the discovery of new lipases and the elucidation of their three-dimensional structures has demonstrated that these two features are not universally present. Lipases from Pseudomonas aeruginosa and Candida Antarctica do not exhibit interfacial activation despite the presence of the lid. Thus, the ability of lipases to hydrolyze carboxylic esters of long chain.from triglycerides distinguishes them from esterases, whereas esterases activity is limited to carboxylic esters of short chain.Although not all lipases exhibit interfacial activation, lipolysis occurs primarily at water/lipid interfaces, so their kinetics cannot be described by the Michaelis-Menten model, which describes reactions in a homogeneous medium and assumes that the enzyme and substrate are soluble in the medium. In this way, kinetic models for lipase-catalyzed reactions should account for the need for the water/lipid interface to form. Verger and Haas.proposed a lipolysis reaction kinetics.This model is divided into two stages, the first of which is the sorption capacity of the enzyme (Figure 3.1).

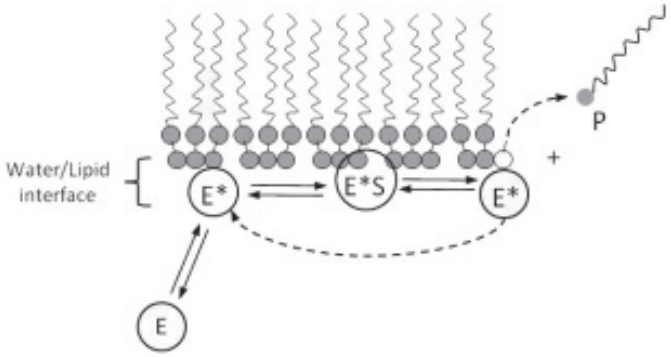

Figure 3.1 Represent the Kinetic model for lipase activity

In the second step, the balance follows the Michaelis-Menten kinetic model, in which the enzyme adsorbed at the interface.binds to the substrate.to form the enzyme-substrate complex, then proceeds to form the product.and enzyme regeneration in their adsorbed form. However, because the substrate concentration there in adsorbed lipase neighbourhood is a concentration on the surface of the water/lipid interface, it must be expressed per area unit rather than per volume unit. This kinetic model also explains how lid affects lipase interfacial activation.

When an amphiphilic sequence interacts with a hydrophobic surface at the water/lipid interface, a conformational change occurs, allowing the active site of the enzyme to access hydrophobic substrates. The opening of the active site to hydrophobic substrates at the interface, in turn, results in a significant increase in hydrolysis activity, which responds by interfacial activation.In addition to hydrolyzing the ester linkage between long-chain fatty acids, glycerol lipases have chemo and region selectivity, starting to act on specific fatty acids and in specific positions on the glycerol molecule, respectively. Because of the differences in fatty acid type and position, lipases are useful tools for modifying the physical, chemical, and nutritional properties of lipids found in food, allowing for manipulation of sensory attributes and nutritional quality.

In the food industry, lipase from Rhizomucor miehie is used to modify cocoa butter by replacing a palmitic acid molecule with a stearic acid molecule, resulting in a triglyceride containing stearic-oleic-estearic acids. This modification raises the natural cocoa butter's melting temperature.and eliminates the unpleasant sensory characteristic of melting in the mouth. Lipases can also be used to enrich oils and fats with unsaturated fats acids, which are necessary for human nutrition.

In terms of chemo and regio selectivity, lipases are classified according to their ability to hydrolyze fatty acids with short, medium, or long chains, as well as their ability to hydrolyze fatty acids linked to Positions 1 and 3 of glycerol or attached to Position 2. Lipases with varying properties result from the combination of these properties. Song *et al.* classified lipases from nine microorganisms into four groups using hierarchical clustering analysis. Lipases specific for esters of medium-chain fatty acids with no hydrolysis at position 2 of glycerol.and lipases specific for esters of short-chain fatty acids with hydrolysis at position 2 of glycerol.were considered the most important. The majority of these enzymes are lipases, which are used in the dairy sector and whose primary activity is the hydrolysis of short- and medium-chain fatty acids.Regardless of lipase substrate specificity, all microbial lipases use the same mechanism of action to hydrolyze fatty acids from glycerol and have the same basic structure in the active site, which consists of a catalytic triad of the amino acids serine, aspartic or glutamic acid, and histidine organised into a -turn- motif that includes the conserved pentapeptide GX1.

The hydrolysis of the fatty acid-glycerol ester bond begins with the binding of the catalytic serine to carbonyl in the lipid ester bond, forming a tetrahedral intermediate. The other catalytic residues, His and Asp/Glu, stabilise this intermediate, and the ester bond is cleaved with lipids esterified to catalytic serine. The ester bond between the serine and the fatty acid is then hydrolyzed by one molecule of water, forming another tetrahedral intermediate from which the fatty acid is released.

Despite the fact that they use the same basic catalytic mechanism,The physicochemical properties of lipases vary greatly, with temperature and pH being two of the most important characteristics for using these enzymes in food processing and production.In the food industry, for example, processing weather conditions and pH levels are relatively mild, and these enzymes should perform optimally under these conditions.The majority of lipases used in food production are thermophilic, with optimum activity at temperatures above 40°C and PhS of action near neutrality. Food processing at high temperatures reduces the risk of contamination with mesophilic microorganisms, reduces feedstock viscosity, and increases reaction rate. The presence of cations in the reaction medium is also important for lipase activity.In general, Ca^{2+} stimulates lipase activity, which helps to explain the success of lipases in the dairy industry. Cations such as Co^{2+}, Ni^{2+}, Sn^{2+}, and Hg^{2+}, on the other hand, are strong lipase inhibitors, meanwhile Zn^{2+}, Mg^{2+}, EDTA, and SDS result in moderate inhibition of these enzymes.

Lipase in aromatic compounds

In the food industry, the production of minimal esters, such as aromatic compounds, is critical. In this context, the demand for esters and aromatic aroma for the food, cosmetics, and pharmaceutical industries ranks fourth among the most commonly used food additives. Direct extraction of fruit, enzymatic synthesis, or chemical synthesis using esterification reactions in the presence of inorganic catalysts at elevated temperatures can all yield low-molecular-weight ester. However, esters obtained through chemical synthesis are considered "unnatural" and have a lower market value than natural esters. When compared to chemically synthesised esters, the use of enzymes for ester synthesis is an alternative solution for obtaining 'natural' compounds that retain odour.

The rose aromatic ester 2-phenylethyl acetate.is widely used in cosmetics, soaps, food, and beverages. Kuo and colleagues.described the enzymatic synthesis of 2-PEAc via vinyl acetate methyl ester with 2-phenethyl alcohol catalysed by *Candida antartica* immobilised lipase. Terpene esters of fatty acids are essential oils that are used in the pharmaceutical, cosmetic, and food industries. The most important are the acyclic terpene alcohols, geraniol and citrollenol. Surprisingly, the use of an Aspergillus lipase. It has already been reported that niger can synthesise terpene alcohol esters of short chain.and fatty acid esters Indeed, several citronelil esters.have been synthesised using *Aspergillus niger* immobilised lipase

Ferraz and colleagues also investigated the production of geranyl propionate using non-commercial immobilised lipase from *Penicillium crustosum*. Lipase from Pseudomonas strains P38 was used by Joseph and colleagues.to synthesise n-heptane butyl caprylate flavouring compounds. In summary, these findings show that lipases have a great potential for producing several aromatic compounds that are commonly utilized by the food industry.

Lipase in dairy products

Lipases are used in the dairy industry to break down milk fat, altering the length of fatty acids and increasing flavour production during cheese ripening.Pancreatic enzymes and gastric tissue lipases from youths ruminants are traditional sources of lipases for enhancing cheese flavour.

Microbial lipase is also used in the production of high-quality cheese. Microbial amylases used in cheese production include the following.

Lipases from Yarrowia lipolytica are thought to be important for cheese maturation because they release fatty acids.that are responsible for the cheese's sensory characteristics. Butanoic acid, for example, contributes to the flavour of Cheddar and Camembert cheese. Lipase from Checking and rechecking niger was tested for its ability to accelerate cheese ripening in an encapsulated form.

Lipase in oils and fats industry

One of the primary areas in the food-processing industry that requires novel economic and green technologies is fats and oil modification. Lipases are popular because of their ability to specifically catalyse hydrolysis reactions such as esterification and interesterification.Lipases are enzymes that can change the location of the fatty acid chains in the triglyceride molecule and/or exchange one or more fatty acid chains with a new molecule.Lipases could be used to produce oils with specific compositions without the use of chemical catalysts that could contaminate the final product, resulting in a higher value-added product while minimising environmental impact.

Following evidence of the negative effects of trans-fats on human health, the partial hydrogenation process used to make margarine, for example, has been replaced by other oil and fat modification methods such as fractionation, interesterification, and full hydrogenation. Catalysis alkaline or enzymatic can promote interesterification, which involves rearranging the fatty acid chains in a triacylglycerol. The alkaline catalysis produces a mixture of triglycerides in which the fatty acids are randomised, whereas the catalysis enzyme with lipase is specific when changing positions 1 and 3, without affecting the fatty acid binding in position 2 of glycerol.

The interesterification of vegetable oil produces the 'hard stock,' which is the main component of margarine. The conventional interesterification process takes place at high temperatures and is catalysed by an inorganic sodium methoxide catalyst. The process consumes a lot of energy and generates byproducts that are removed in a series of bleaching steps. An immobilised lipase could also be used as a catalyst in the interesterification of oils. The process is highly specific, operates at a low temperature, and produces fewer by-products.

Because of its high stability when immobilised, lipase from Thermomyces lanuginosus, commercially available as TLL (IM Lipozyme TL), is frequently used in oil and fat. TLL has been used to hydrolyze oils and fats in order to obtain fatty acids and free glycerol (Fernandez-Lafuente, 2010).Other uses for lipases include the hydrolysis of fish oil to obtain polyunsaturated fatty acids. Lyberg and Adlercreutz (2008) investigated the specificity of five lipases in the hydrolysis of EPA and DHA present in fish oil in a system containing methyl ester of EPA, DHA, and palmitic acid. The authors found that EPA and DHA in fish oil had higher hydrolysis activity than methyl esters. Among the lipases tested, the TLL and one lipase from *Candida rugosa* performed the best.

Furthermore, commercially available lipase from *Rhizomucor miehei* (RML) has been reported to be effective in the hydrolysis of fatty acids. Although RML is a sn-1, 3-specific lipase,

spontaneous acyl migration from position 2 to positions 1 or 3 allows for full triglyceride hydrolysis. According to studies, the enzyme performed well in the hydrolysis of various oils, including peanut oil, which contains a high concentration of polyunsaturated fatty acids in position sn-1,3. Sovavá and colleagues investigated the hydrolysis activity of RML on blackcurrant seed oil (rich in linoleic and linolenic acids) to obtain different levels of - and/or - linolenic acid in the mixture of liberated fatty acids and the fraction of di- and monoacylglycerols, making them suitable for special dietary needs. The results showed that liberated fatty acids had higher levels of -linolenic, palmitic, and stearic acids, whereas di- and monoacylglycerols had higher levels of -linolenic and stearidonic acids.

In nature, the esterification reaction is thought to be the inverse of the lipase reaction. This reaction is characterised by the direct condensation of a fatty acid and an alcohol, which could be glycerin, a mono- or diglyceride, or another alcohol (Rodrigues and Ferandez-Lafuente, 2010). One example is the deacidification of palm oil by *Rhizomucor miehei* lipase (Kumar and Krishna, 2014). Lipase, ethanol, and sodium hydroxide were used to deacidify crude red palm oil with an 8.7% free fatty acid content.Oil deacidification using enzyme yielded nearly 100% product yield. When compared to the other two methods of deacidification, the enzyme deacidified oil had a higher value in unsaponifiable matter (0.91%), monoacylglycerols (2.8%), and diacylglycerols (18.7%).

Milk fat is an excellent source of essential fatty acids and fat-soluble vitamins. Milk fat, because of its versatility and complexity, offers both opportunities and challenges for modifying its composition for various applications. Tecelo and colleagues investigated the ability of Rhizopus oryzae lipase (rROL) to synthesise triglyceride substitutes in human breast milk. When rROL was immobilised in lewatit and accurel, it showed a percentage of oleic acid incorporation of approximately 30 and 22 mol.per cent, respectively. The commercial lipase RML-Lipozyme RM IM was also used successfully in the incorporation of oleic acid in triglyceride fat milk.

Lipases from yeast and filamentous fungi with high specificity have multiple applications in the dairy industry, such as low-calorie milk fat synthesis. Lipases from Candida Antarctica (Novozyme 435) and Rhyzomucror miehei (1M RM) have been identified as important enzymes for the synthesis of di- or monoglycerides with unique compositions. The incorporation of linoleic acid into dairy foods by immobilised lipases is also described in the literature.Omega-3 fatty acids have been shown to have numerous health benefits. EPA and DHA are the two most important omega-3 long chains, with the latter being required for brain development and foetal retina (Swanson et al., 2012). Milk fat can be fortified with omega 3 (linolenic acid), a precursor in the metabolism for the formation of EPA and DHA. Faustino *et al.* (2015) investigated the efficacy of non-commercial heterologous lipase from Rhizopus oryzae (rROL) immobilised on Lewatit VP OC 1600 or Relizyme OD403/S and commercial lipase from *Rhizomucor miehei* (Lipozyme RM IM) in the incorporation of omega-3 linolenic acid in tripalmin.Using Lipozyme RM IM and rROL immobilised on Lewatit or Relizyme, the authors observed 48.9, 43.6, and 18.3 mol. percentage fatty acid incorporation in triacylglycerols (TAG). As an alternative to commercially available immobilised lipases, rROL immobilised on Lewatit was chosen.

Cocoa butter fat is an important byproduct of chocolate production, but it is frequently in short supply and its price varies greatly. As a result, cocoa butter fat substitutes are extremely important in the food industry. Triglycerides esterified with saturated fatty acids (palmitic and stearic acid) at position sn-1,3 and monounsaturated fatty acid (oleic acid) at position sn-2 make up the majority of cocoa butter.

This structure is responsible for the rheological and sensorial properties of cocoa butter. Lipases can catalyse the transesterification of cheap oils and one fraction of palm oil (pal mid fraction) to produce cocoa butter. Unilever and Fuji Oil have developed a large-scale enzymatic process for producing a substituent lipid matrix cocoa butter. Acidolysis of refined palm oil and palmitic, stearic acids using the commercial immobilised lipase IM Lipozyme from Mucor miehei produced cocoa butter equivalent. The process produced cocoa butter equivalent with melting temperatures ranging from 34.7 to 39.6°C, which is comparable to cocoa butter.

Monoglycerides (MG) are amphipathic molecules that are widely used in the food industry as emulsifying agents. They are obtained through the chemical catalysis of glycerolysis of fats or oils at high temperatures (220-250oC), which consumes a lot of energy and results in a dark-colored product due to by-product formation. Furthermore, the chemical process produces a mixture of MG (5-60%), diacylglycerols (DG) (35-50%), triglycerides (1-20%), free fatty acids (1-10%), and 230 Microbial Enzyme Technology in Food Applications alkali metal salts. The ideal would be to obtain an MG content of 80-90 percent. Then, several studies were conducted to produce MG by esterifying glycerol with palmitic acid. Freitas *et al.* (2010) reported that a lipase from Penicillium camemberti immobilised on an Epoxy-PVA-SiO2 composite produced 46% of MG, 6% of diacylglycerols, and 3% of triglycerides.Lipases from the Penicillium genus, such as *P. roqueforti*, *P. camembertti*, *P. expansum*, and *P. abeanum*, are extremely capable of modifying oils and fats (Li and Zhong, 2010). Kapoor and Gupta (2012) studied different formulations of lipase B from *Candida antarctica* (CLEAS: cross-linked enzyme agregates; PCMS: protein coated microcrystals; CLPCMCs: cross linked protein coated microcrystals) and found 81 per cent of MG and 4.5 per cent DG using the CLEAS formulation; 82 per cent of MG and 4 per cent of DG with PCMCs; and 87 per cent de MG e de 3,3 per cent de DG using the CLPCMs formulation.

Lipases in Bakery

Bread, cake, sweets (pastries), crackers, cookies, pies, and tortillas are made up of carbohydrate (flour and sugar), protein (wheat flour and eggs), and lipids (flour, eggs, emulsifiers, margarine and butter). Lipids play an important role in the baking and storage processes. Furthermore, they can be converted enzymatically into useful emulsifiers for bread dough.Lipases, in this context, strengthen mass stability, increase loaf volume, and improve texture and shelf-life.

Wheat flour lipids are classified as amide lipids, which are found in starch granules, and non-starchy lipids, which can be found free or bound to other flour constituents as proteins. Polar starch granules can be extracted using polar solvents such as butanol, whereas non-starchy lipids can be extracted using non-polar solvents such as n-hexane. In the bakery, surfactants such as diacetyl tartaric esters of mono- and diglycerides (DATEM), sodium stearoyl lactylate (SSL),

and monoacylglycerols are used. These surfactants are essential for stabilising the gas-liquid interfacial interaction during the backing fermentation process.In comparison to other enzymes such as -amylase and proteases, the use of lipase in baking is relatively new. The first generation of commercial lipase preparations hit the market in 1990, and they were used to improve dough stability and volume. In comparison to the first generation lipases, the second generation lipases produce more polar lipids, provide a greater increase in volume, better stability to mechanical stress on the dough, and a fine, uniform bread crumb structure.

A third generation lipase was recently discovered to expand the gluten network, increase wall thickness, and reduce cell density, thereby improving the volume and crumb structure of high-fiber white bread.

Lipase functionality in bread manufacturing is associated with the hydrolysis of one or more fatty acids from nonpolar triglycerides and/or polar lipids (phospholipids and glycolipids) to form polar mono- and diacyl-forms. These products were investigated due to their significant emulsifier properties and functionality similar to DATEM, which has been widely used as dough conditioners and/or crumb softeners in breadmaking.There are three possible explanations for the recent interest in lipases in the baking industry.First, lipases may replace traditional surfactants by producing surfactants in situ via hydrolysis of endogenous lipids. This method would lower the costs of production, storage, and transportation. Second, due to denaturation during the baking process, lipases are not detected in the final product. Third, lipases are easily produced at room temperature and can be heterologously expressed.Olesen et al., described the use of microbial lipases to improve dough properties. This patent discloses a method for improving dough properties by adding microbial lipases from *Humicola lanuginosu*, *Rhizomucor miehei*, *Pseudomonas cepacia*, and *C. Pastase*, *Rhizomucor miehei*.

The effect of four commercial microbial lipases on bread volume was evaluated: YieldMAX (phospholipase A1 from *Fusarium venenatum*), Lipolase (lipase from *Thermomyces lanuginosus*), Lecitase Ultra (resulting from the combination of phospholipase T. lanuginosus gene and Fusarium oxysporum), and Lipopan F (an enzyme preparation of Fusarium oxyspor.Lipases increase loaf volume due to stabilised gas cells during fermentation and require a balance of different types of lipids. Enzymes increased the volume of bread by altering the lipid composition of wheat flour by lowering the levels of galactolipids and phospholipids while increasing the levels of lysolipids and free fatty acids. After treating bread flour with two different commercial microbial lipases, Schaffarczyk and colleagues confirmed the importance of lipid composition and performance of bread flour during the baking process. The changes induced by lipases in the lipid fraction caused an increase in bread volume of 56-58 percent, depending on the type and concentration of added lipase.

Lipases in wine

Wine is a complex blend of thousands of compounds that contribute to its colour, flavour, and aroma. Wine aroma research has received a lot of attention in recent decades, and a lot of sensory compounds have been identified. Among these compounds, ethyl esters have gotten a lot of attention because of their strong influence on taste. The most common compound found in

wines is ethyl acetate ester. However, other esters have been reported in the literature, including 2-ethyl hidroxpropionato, diethyl butanediato, ethyl butanoate, ethyl hexanoate, octanoate, ethyl decanoate, ethyl 2-methyl-propionate, ethyl 3-methylbutanoate, ethyl cinnamate, methyl-but.

Wine esters are divided into two types: those formed enzymatically during the beverage's ageing process and those formed by chemical esterification between an alcohol and an acid. Together with esterases, lipases with specific properties, such as high activity on pH 5-7, stability in the presence of ethanol, sodium metabisulfate, malic, tartaric, citric and lactic acid, can be used to produce ethyl acetate, ethyl butanoate, ethyl hexanoate and ethyl.Esteban-Torres and colleagues (2014) investigated the properties of lipase/esterase produced by Escherichia coli BL21 that had been genetically modified by the insertion of a gene encoding the lipase/esterase enzyme derived from Lactobacillus plantarum WCFS.

The enzyme produced by the microorganism demonstrated a high potential for use in the winemaking process, with high activity at low pH and stability in the presence of ethanol, sodium metabisulfite, tartaric, lactic, and citric acids. Lee and colleagues investigated the effect of sequential inoculation of yeasts, Williopsis saturnus var. mrakii NCYC2251 and *Saccharomyces cerevisiae* var. bayanus R2, on ester production during papaya wine processing. Sequential inoculation of non-Saccharomyces and Saccharomyces yeasts, according to the authors, may be a useful tool for manipulating yeast succession and modulating the volatile profiles and organoleptic properties of papaya wine. Lipases could also be used to remove fat from meat and fish products in a process known as biolipolysis. They are also important in the fermentation of sausage and are used to determine changes in long-chain fatty acids during ripening.

The use of lipases in the refining of rice flour, the modification of soy milk, and the improvement and acceleration of the fermentation process of rice wine were also discussed. Immobilized lipases were also used to esterify phenols from functionalized vegetable sunflower oil for antioxidant synthesis. The use of lipases as antioxidants for oil incorporation was also mentioned. Lipases have also been used in the processing of black tea to degrade membrane lipids and initiate the formation of volatile flavouring in any product.

Microbiological enzymes in food technology

Enzymes are critical for the survival of all living things. In chemical reactions, they act as catalysts. Microbial enzymes are crucial in the development of industrial bioprocesses because they act as metabolic catalysts. Enzymes have been used in food preservation for millennia, and today they enable various food industries to provide product quality and stability while increasing production efficiency. Microbial enzymes in culinary purposes have not only helped to diversify the food industry, but have also generated economic assets. Since ancient times, the increasing demand for sustainable food has fueled the use of microbial enzymes, whether knowingly or unknowingly. Microorganisms have always been the most abundant and beneficial source of many enzymes. They also offer environmentally friendly products to customers, reducing energy, water, and raw material consumption while producing less waste. Enzymes

help industrial processes by reducing energy consumption and increasing efficiency while also contributing to their sustainability.

Although not in isolated form, enzymes have been used for centuries in dairy, baking, brewing, and winemaking. Their applications keep bread soft and fresh for an extended period of time, increase dough volume, and provide a crispy crust. Enzymes have been used in beer and wine for centuries to reduce calorie and alcohol content, as well as to improve clarity and flavour. Though used unknowingly for centuries, the use of enzymatic or a whole microbial cell as a biocatalyst has established a revolution in the food industry.The microbial enzyme is widely used in industry and commerce. Microbes have proven to be the most effective and abundant source of enzymes. The current article discusses major advances in the production of essential microbial enzymes and their applications in the food industry.

Catalyzes

Biocatalysts are the most capable biological macromolecules produced by living organisms, and they provide more competitive processes than chemical catalysts. These are proteins that accelerate chemical reactions and may cause electron or functional group transfers to produce a specific biochemical reaction. They are in charge of all critical chemical interconversions required for life to exist. Because of advancements in recombinant technology, protein engineering, and computational design, applications of tailor-made biocatalysts with desired properties in various industries have increased in recent years.

Catalases are one of the microbial enzymes that are in high demand in the industrial sector. The global industrial enzyme market is rapidly expanding, with a total turnover of approximately $3.3 billion in 2010 and expected to reach $7.1 billion in 2018. (BCC Research Report, 2014). Catalases (EC 1.11.1.6) are a type of enzyme. The most common industrial enzymes, with the highest turnover and a wide range of industrial, diagnostic, and medical fields (Sooch et al., 2014b). Catalase is a haem-containing enzyme in the Oxidoreductases class. Catalases were among the first enzymes discovered in biochemistry (Loew, 1900). They convert hydrogen peroxide (H2 O2) to oxygen and water and are an important component of the cell's defence mechanism against peroxidation.

They can be found in a wide variety of plants, animals, and microorganisms. Catalases are known to cause a variety of redox reactions in addition to catalysing the decomposition of hydrogen peroxide (H_2O_2). The precise mechanisms for oxidase and peroxidase activities, however, have yet to be determined experimentally. Catalases are being explored and used for a variety of applications while mechanistic studies are being conducted. Catalases are found in peroxisomes, eukaryotes, and.However, cytosolic catalases have been found in eukaryotes such as.Caenorhabditis elegans, and Neurospora crassa.Catalases have shown promise in a variety of applications in recent years. Microbial catalases are preferred because of their economic feasibility, high yield, ease of product modification and optimization, consistent supply due to the absence of seasonal fluctuations, and rapid growth of microbes on low-cost media. However, it is worth noting that most commercial catalases have optimal activity at temperatures ranging from 20°C to 50°C and at neutral pH, making them unsuitable for use in some industrial

processes. Despite their high potential, catalases' industrial applications have been hampered by undesirable properties such as low stability, low catalytic efficiency, and low specificity. A variety of approaches have been tried to overcome such shortcomings, including screening of enzymes from natural sources, designing microbes with recombinant technologies, random mutations, and immobilisation.

CHAPTER 4

CLASSIFICATION AND STRUCTURAL ASPECT OF MICROBIAL CATALASES

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Monofunctional haem catalases are members of the original class of catalases found in all animals, plants, and microorganisms. The structure of mono-functional catalases differs depending on the number and identity of domains in different organisms. Catalases are typically found as a barbell tetramer of four identical subunits. These catalases have a molecular mass ranging from 200 to 340 kDa and a haem prosthetic group at the catalytic centre. The haem group, which is located between the internal walls of the beta barrel and several helices, is responsible for catalase enzymatic activity. At least two gene duplication events resulted in the division of haem catalases into three clades. Clade 1 catalases have about 500 residues per subunit and are mostly of plant origin, with a bacterial subgroup. Clade 2 catalases, which have about 750 residues per subunit, are mostly bacterial or fungal in origin. Archaea, bacteria, fungi, and some eukaryotes contain clade 3 catalases with nearly 500 residues per subunit.

The absence of these older taxonomic groups of catalases in organisms suggests that they evolved later. Catalase-peroxidase is a less common type of catalase that belongs to the second group of catalases. These are only found in aerobic bacteria and have a molecular weight of 120-340 kDa. Catalase-peroxidases are similar to plant and fungal peroxidases but do not exist in plants or animals. These bi-functional catalases, like haem-containing catalases, have peroxidative activity in addition to catalytic activity (Welinder, 1991; Kapetanaki et al., 2007). The presence of a covalent adduct in which tyrosine is attached at its ortho position with methionine on one side and a tryptophan is linked on the other side is a distinguishing feature in most catalase-peroxidase structures.

Non-haem catalases are a minor bacterial protein family that has manganese in the active site rather than a haem molecule and is also known as pseudocatalase or manganese catalase. These have a molecular weight of 170-210 kDa. The manganese catalase sequence is distinguished by the presence of highly conserved essential ligands (glutamate and histidine). Because of lateral gene transfer between different bacterial taxa, these non-haem catalases are classified into five distinct clades. Clade 1 consists of archaebacteria and at least one Firmicutes, whereas Clade 2 consists of Actinomycetes and Firmicutes. Clade 3 is found in Proteobacteria and Clostridia. Clade 4 is dominated by the Bacteroides genus, as well as many Cyanobacteria, whereas Clade 5 is dominated by Proteobacteria. Clades 1 and 2 have a common ancestor, whereas Clade 5 is the most distantly related to the other clades.

The fourth minor class of catalases includes several haem-containing proteins with very low catalytic activity. They include bi-functional enzymes such as chloroperoxidases,

bromoperoxidases, and catalase-phenol oxidases. Catalase-phenol oxidase is a tetrameric haem protein with a molecular mass of 320 kDa, whereas chloroperoxidase is a 42 kDa monomer (Kocabas et al., 2008). Catalase-phenol oxidase is a catalase-like enzyme that can oxidise a variety of phenolic compounds in the absence of hydrogen peroxide.

As a Biosensor

Sezginturk and colleagues (2005) developed an immobilised catalase-based Clark-type electrode for the detection of the highly toxic chemical azide in fruit juices such as black cherry juice, orange juice, and apricot juice, which can detect azide in the range 25 M to 300 M. Similarly, a polyaniline-based catalase biosensor was built to detect hydrogen peroxide and azide in various biological samples.Akertek and Tarhan investigated the use of immobilised catalase from Aspergillus niger on modified SiO2 support in a batch-type reactor for removing traces of hydrogen peroxide used during milk pasteurisation (1995). To determine the decomposition level of hydrogen peroxide in milk samples in dairy industries, a catalase-based biosensing system was built by immobilising enzyme on a dissolved oxygen probe membrane with the help of gelatin. Akyilmaz and Kozgus created another catalase-based amperometric bio-device for detecting calcium in milk and water samples by immobilising enzyme on a Teflon membrane. This bio-device had a detection limit of 1 mM to 10 mM and a response time of one minute. Based on the enzyme activity of catalase in infected milk samples, a quick and simple biosensor-based method for detecting mastitis infection in milk was developed. Hnaien *et al.* pioneered the development of a conductimetric biosensor for the detection of ethanol in foods by co-immobilizing catalase and ethanol oxidase. Aruldoss and Kalaichelvan (2014) also created an amperometric catalase-based biosensor for estimating the concentration of alcohol in alcoholic beverages. This bio-device was also capable of determining calcium levels in cow milk samples in 3 minutes. Kroll and colleagues developed a catalase-based method for assessing bacterial colony contamination in food samples with a detection limit of 103 cells/mL (Figure 4.1).

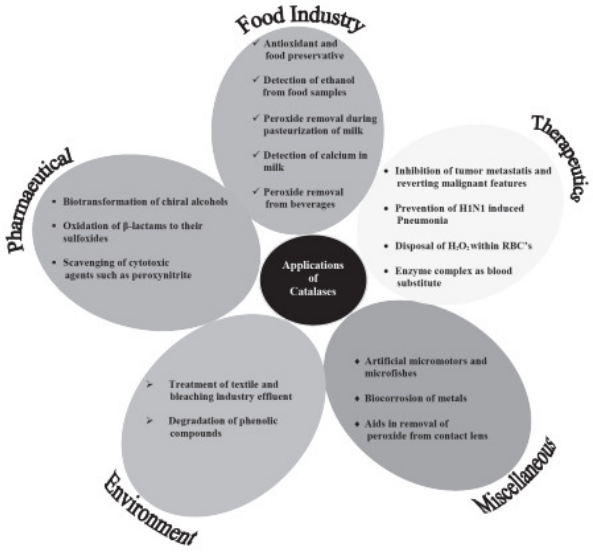

Figure 4.1 Represent the Applications of catalases.

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To determine the decomposition level of h2so4 in milk samples in dairy industries, a biosensing system was created by immobilising enzyme on a dissolved oxygen probe membrane with the help of gelatin. Akyilmaz and Kozgus created another catalase-based amperometric bio-device for calcium detection in milk and water samples by immobilising enzyme on a teflon membrane. This bio-device has a detection limit of 1 mM to 10 mM and a response time of one minute. Based on enzyme activity of catalase in infected milk samples, a quick and simple biosensor-based method for detection of mastitis infection in milk was developed. Hnaien *et al.* pioneered the development of a conductimetric biosensor for detecting ethanol in foods by co-immobilizing catalase and ethanol oxidase. Aruldoss and Kalaichelvan also created an amperometric catalase-based biosensor for estimating alcohol concentration in alcoholic beverages. This bio-device was also capable of determining calcium levels in cow milk samples within 3 minutes. Kroll and colleagues developed a catalase-based method for assessing.

With increased awareness of nutritional needs, bifunctional catalases with additional peroxidase activity have received a lot of attention. The use of catalase in conjunction with oxidase aids in the scavenging of free radicals, thereby avoiding oxidation, which is responsible for food deterioration. Dondero *et al.* investigated the preservative influence of catalase (Cat) and glucose oxidase (Gox) in 4% (w/v) glucose (Glu) solution on fishes and shrimps (1993). It was discovered that soaking shrimp and fish in Cat/Gox/Glu solution delayed microbial spoilage and increased product freshness. Based on these findings, it was hypothesised that the active oxygen species produced by these enzymes acted as bactericides, thereby extending the shelf life of food.

Catalase-based Pharmaceuticals

The search for green technologies for the synthesis of pharmaceutical substances has intensified in recent years. As a result, the industry is looking for low-cost and environmentally friendly biocatalytic pathways to replace traditional chemical processes. Catalases have been used in the pharmaceutical industry for a variety of redox reactions. The purified catalaseperoxidase from Bacillus pumilus was used to oxidise antibiotic pharmacophores (i.e., -lactams) into their sulfoxides in less time than the chemical route with enantio pure building blocks. A biocatalytic method for stereo-selective oxidation of pharmacophores was also developed, with potential

applications in the treatment of bacterial infections. Magner and Klibanov investigated the catalase-mediated enantio-selective biotransformation of some chiral alcohols in organic solvents (2, 3-butanediol, trans-1, 2-cyclohexanediol, trans-3-methylcyclohexanol, menthol, 2-methyl-1-butanol, etc.). Catalase's catalytic role in decay.

Catalases in Bioremediation

Environmental legislation restricts the discharge of H_2O_2-containing industrial effluents into open streams. In the textile and paper industries, H_2O_2 is commonly used as a bleaching agent. Traditional methods of removing unused H_2O_2 include further washing of bleached fabrics, which generates large amounts of alkaline waste water. Alternatively, H_2O_2 can be removed using chemicals such as sodium bisulphite, which results in high salt levels in the process streams. Catalases are attractive eco-friendly alternatives for H_2O_2 removal due to their high catalytic rates. Several researchers have successfully used catalase enzyme to remove hydrogen peroxide from textile and bleaching industry effluents in order to reduce pollution load.

The dyeing quality of cotton fabrics improved after the hydrogen peroxide residues were removed with catalase. Paar *et al.* used immobilised thermoalkalistable catalase from Bacillus sp. to degrade hydrogen peroxide in textile bleaching effluent, allowing treated water to be reused for dyeing. Catalase-producing alginate entrapped Bacillus sp. TE-7 cells were used in another study to degrade H2 O2 in a packed bed reactor (Sooch and Kauldhar, 2015). The action of catalaseperoxidase from Comamonas terrigena N3 H on phenolic compounds has been reported.

Laccase

Laccase enzymes (p-diphenol oxygen oxidoreductases EC 1.10.3.2) are copper-containing oxidases that are abundant in nature. After discovering that the use of so-called redox mediators can extend the catalytic action of these almost-forgotten enzymes, interest in them skyrocketed. This feature, along with its broad substrate specificity and the fact that it only requires oxygen (which is easily obtained from air), makes laccases very appealing for a wide range of industrial and biotechnological applications, which would include food processing.

The only microorganisms that degrade the bulky, recalcitrant, and heterogeneous polymer lignin are white-rot fungi. This ability is due to the secretion of an extracellular and non-specific enzymatic complex during secondary metabolism, which is usually triggered by nitrogen deficiency. This enzymatic complex is composed primarily of lignin peroxidases, manganese-dependent peroxidases, and laccases, as well as other complementary enzymes. Peroxidases are more powerful oxidants than laccases, but they require hydrogen peroxide to catalyse their activity. Furthermore, MnP dependence on Mn2+ and LiP dependence on veratryl alcohol limit their practical application. Laccases, on the other hand, require only oxygen (which is readily available from air) to catalyse, producing water as the only by-product, which qualifies them as 'green' catalysts. Laccases are promising biocatalysts for a variety of industrial applications, including bio-bleaching, bio-pulping, wine stabilisers, biodegradation of organic contaminants, textile decoloration, bio-fuel cells, biosensors, antibiotic and anti-cancer drug production, polymer and fibre surface modifications, and so on (Rodriguez-Couto and Toca-Herrera, 2006; Medhavi and Lele, 2009). Among these applications, the ability of laccase to degrade lignin

opens up new possibilities for lignin valorisation, which is currently a hot research topic. Laccases are also excellent candidates for food industry applications because many of their substrates (e.g., unsaturated oils, phenols, thiol-containing proteins) are found in a variety of foods and beverages.

Laccase Producing Microorganisms

Laccases are common enzymes found in nature. Laccase was discovered in the exudates of the Japanese lacquer tree, Toxicodendron vernicifluum (formerly Rhus vernicifera), which gave rise to the name laccase. It was discovered in fungi a few years later. Since then, they've been discovered in a variety of plants, fungi, bacteria, and insects. Azospirillum lipoferum, a plant-root-associated bacterium, discovered the first bacterial laccase.

Laccase activity was later discovered in other bacteria such as Marinomonas mediterranea, Mycobacterium tuberculosis, and Bacillus sphaericus and *B. subtilis* coat protein A (cotA) endospores. Although extracellular bacterial laccases have been discovered, the majority of the studied bacterial laccases are intracellular. Laccases are particularly abundant in white-rot fungi, which are the only microorganisms known to degrade the entire wood component (cellulose, hemicellulose, and lignin). Laccases are produced to varying degrees by almost all white-rot fungi. Trametes versicolor (formerly known as Coriolus versicolor or Polyporus versicolor) is the most studied fungus for laccase production.

Catalysis Mechanism

Laccases are copper oxidases that have multiple functions. They have four copper atoms at their active site, which are distributed in one mononuclear cluster called T1, which is responsible for the enzyme's blue colour, and one trinuclear cluster called T2/T3 (Mayer and Staples, 2002). Laccases that lack the T1 copper are referred to as yellow or white laccases. The electrons involved in the reaction are transferred. The 'blue' copper (T1 site) is thought to accept electrons from the reducing substrates, which are then transmitted to the tri-nuclear copper cluster (T2/T3), where oxygen is reduced to water. As a result, four substrate molecules are oxidised into four radicals, while one oxygen molecule is reduced into two water molecules (Morozova et al., 2007). The redox potential of the T1 copper site is directly responsible for the enzyme's catalytic capacity. Laccases, unlike most enzymes, have a low substrate specificity, allowing them to degrade compounds with lignin-like structures such as polyaromatic hydrocarbons (PAHs), textile dyes, and other xenobiotic compounds, and thus their industrial interest. However, the range of substrates susceptible to oxidation by laccases is limited by their low redox potential, because oxidation depends on the redox potential difference between the substrate and the T1 copper of laccase (Xu, 1996). The T1 copper of laccase has redox potentials ranging from 0.4 V to 0.8 V versus a normal hydrogen electrode (NHE). As a result, if the substrate of interest has a particularly high redox potential, such as non-phenolic lignin units, laccase is unable to directly oxidise it. Also, the substrate of interest could be a compound that does not fit into the active centre of laccase due to size or steric hindrance. These constraints can be overcome by employing so-called redox mediators. The redox mediators are low-molecular-weight organic compounds that are oxidised by laccases, forming highly reactive cation radicals that oxidise

compounds that laccases alone cannot. Figure 2 depicts a laccase catalysis scheme with and without redox mediators. There are over 100 redox mediators described, but the most common are 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 1-hydroxybenzotriazole (HBT).

However, these synthetic mediators are toxic and expensive, prompting the search for natural-source alternatives. According to Caas and Camarero, several lignin-derived phenols are cost-effective and environmentally friendly alternatives to synthetic mediators.Babu *et al.* reviewed the biochemical properties of laccases in depth. Laccases are frequently found as isoenzymes or monomers, which oligomerize to form multimeric complexes. Each isoenzyme has four copper atoms and can perform the reaction mechanism of laccases independently.Laccase monomers have molecular masses ranging from 40 to 130 kDa and are glycosylated to varying degrees in fungi and plants (between 10 and 25% and 20 to 45%). This property is thought to protect laccases from proteolysis and inactivation at high temperatures.

When the substrate is a hydrogen atom donor compound, fungal laccases typically have an optimum pH of 3-5 and bacterial laccases have an optimum pH of 5-6. (e.g., ABTS). When a phenolic compound (e.g., syringaldazine) is used as the substrate, the optimal pH is shifted to 6-7 as a result of the substrate's redox potential balance and the inhibition of the T2/T3 copper site by the binding of a hydroxide anion. The isoeletric points (pI) of fungal laccases range from 3 to 7, whereas the pI of plant laccases is around 9. Fungal laccases are also more stable at higher acidic pH, though there are exceptions.The ideal temperature for laccase is usually determined by the source. Laccases, in general, have optimum temperatures of 30-50°C and rapidly lose activity at temperatures above 60°C. However, temperature stability varies greatly. The redox potential of fungal laccases, on the other hand, ranges from 0.4-0.5 V in plants and bacteria (Gianfreda et al., 1999; Duro et al., 2006) to 0.4-0.9 V in plants and bacteria.

Laccase Production

Laccase is produced using both submerged (Clsm) and solid-state fermentation (SSF) techniques. SmF involves the growth of microorganisms in a nutrient-rich liquid medium with a high oxygen concentration. The main issue with submerged fermentation is uncontrolled mycelium growth, which results in mass and oxygen transfer limitations (Rodriguez-Couto and Toca-Herrera, 2007). This issue can be addressed by employing a pulsed system to control pellet growth or by cell immobilisation.

SSF is the growth of microorganisms in the absence or near absence of a free-flowing liquid, with an inert substrate or a natural substrate (organic material) serving as a solid support. Because it replicates the natural habitat, SSF has been shown to be particularly suitable for the production of enzymes by filamentous fungi. T. pubescens, a white-rot fungus, is grown under SmF conditions and under SSF conditions on an inert support (nylon sponge cubes).Laccase commercialization is hampered by its low yield and high production costs. Attempts have been made in recent years to overcome this problem and achieve low-cost laccase overproduction. As a result, the use of enzyme inducers and agro- and forestry wastes as substrates has yielded promising results in both SmF and SSF conditions.

Another approach that has piqued the interest of many researchers is the selection of a suitable host overproducing laccase, which can be accomplished by isolating and screening new laccase producers or by constructing gene engineering strains.

Removal of aflatoxin B1 from foodstuffs

The most abundant aflatoxin is aflatoxin B1, which has been shown to be highly mutagenic, toxic, carcinogenic, and teratogenic to humans and animals. Aflatoxin contamination of feed and foodstuffs causes significant economic losses as well as health problems. The various physical and chemical methods used to inactivate aflatoxin have proven ineffective and costly.

As a result, an efficient and low-cost method is required. Alberts *et al.* investigated the degradation of aflatoxin B1 by various laccases from different white-rot fungi. Aflatoxin B1 was significantly degraded (87.34%) when treated with pure laccase from T. versicolor and recombinant laccase from Aspergillus niger (55 per cent). Furthermore, its mutagenicity was significantly reduced. Scarpari *et al.* (2014) discovered that treating contaminated maize with T. versicolor culture filtrates containing primarily laccase enzymes reduced the content of aflatoxin B1 significantly. As a result, laccase treatment can be a promising method for removing aflatoxin B1 from foods.

Role of Intrinsic Enzyme.in brewing

Beer is the most popular alcoholic beverage on the planet and has been produced for at least eight millennia. In 2013, approximately 1.89 billion hectoliters of barley beer were brewed worldwide (FAO, 2015). China, the United States, Brazil, Russia, and Germany produced 506, 224, 135, 89, 86 million litres of barley beer each year, accounting for more than 55% of total global production. Surprisingly, beer production has more than quadrupled in the last 50 years (FAO, 2015). The beer market is expected to grow by approximately 1.2 percent per year in the coming years, owing to consumers' increasing purchasing power and changing drinking habits.Beer is said to have originated in Babylon. Malt was used by the ancient Egyptian and Babylonian cultures to make opaque beers 2400 years ago, according to historical records. The Egyptians passed on their craft beer techniques to the Greeks and Romans, and it spread from there to the rest of the world.

CHAPTER 5

BEER INGREDIENTS

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High germination potential, presence of husk, and ability to synthesise critical enzymes during malting, barley (Hordeum vulgare) is the preferred raw material in brewing. Among the various enzymes, the most ROWLFR diastasic activity (DA). The barley caryopsis is germinated under controlled conditions to optimise enzymes. Grain soaking, germination, and kilning are the three basic steps of germination or malting tHE. synthesis of humic acid in the germ activates the grain during soaking.

Because this hormone regulates germination, the various enzymes produced primarily in the germ are affected.6.The aleurone cells produce ODVHVDUH, which begins to degrade from the outer to the inner starchy endosperm. The ideal malting barley is low in protein and produces sound, uniform kernels that recreate when germinated at temperatures above 5°C.

Brewing Adjuncts

Levels of enzymes However, in the presence of diastatic malt, these are converted to sugars and dextrins during mashing. The vast majority of brewing additivesgrits or refined starches made from refined maize (Zea mays) or rice (Oryza sativa). Starches are practically pure starch, whereas refined grits contain endosperm proteins such as prolamins and glutelins. Glucose or monosaccharide syrups are also used as adjuncts, particularly in the production of light or dark beer.

Malt amylases hydrolyze starchy brewing adjuncts into linear and branched dextrins, maltotriose, maltose, and traces of glucose. Dextrins contribute to the typical beer body, whereas simpler carbohydrates are yeast substrates that are converted into ethanol and other organic compounds. The average particle size of most brewing grits is 40 to 60 US mesh, which favours extraction and lautering. Refined grits are preferred because they have more convertible carbohydrates, fewer pigments and oils, and a brighter colour. The beer that results is less prone to oxidation and has improved flavour and colour.

Hops

The main characteristic of European beer is the fact that it is brewed with hops (Humulus lupulus) to impart the characteristic flavour and aroma. For 1 hL sweet wort, 100 to 300 g hops are typically added. Hops are derived from the cones or female flowers of a perennial plant that grows in either cold or temperate climates. Hop plantations are made up of female plants that grow in fields with long posts that communicate with wires that support the vines. Before harvesting, the female mature flowers are green to yellow in colour and contain bittering and

aromatic compounds enclosed in microscopic organelles called lupulins, which are high in resins, phenolics, tannins, and essential oils. After harvesting, the cones are carefully dehydrated at temperatures ranging from 60 to 65oC for an average of 10 hours, or until the moisture content falls to around 8-10%.

To minimise oxidation and degradation of bitter and flavour compounds, shelf-stable dried hops must be stored cold and airtight. Hops serve a variety of functions in the beer system. Because of the appearance of essential oils and resins, they contribute to the typical flavour and aroma. Because tannins and other polyphenols bind protein that cause haze, they help to clarify the beer. Hops also contribute to the typical beer flavour profile because they contain aldehydes, carboxylic acids, and alcohols. The bitter flavour is caused by resins known as humulones or -acids and lupulones or -acids. The essential oils are a complex mixture of over 300 compounds, including terpenes, aldehydes, ketones, and alcohols.

Polymerization of these organic compounds results in the formation of resin bodies. The essential oil replaces the aroma that's also lost during wort boiling. This is why, when wort boiling is stopped, a portion of the total hops are commonly added.Hops are classified into aroma and bittering types based on the oil-to-resin ratio. The majority of commercial varieties are classified as mid-range, high, or super alpha. The majority of hops used in the brewing industry are dehydrated and vacuum packaged as dense pellets. Hop extracts are made by first treating dehydrated cones with ethanol or methylene chloride, then distilling the organic solvent away from the spent hops to solubilize polyphenols and pectins.

Yeast

Yeast (*Saccharomyces cerevisiae*) is crucial in beer production because it is responsible for converting wort into beer. Ingledew thoroughly covers the yeast biochemical pathway (1995). In the brewing process, yeast is chosen based on the desired fermentation power, fermentation temperature, and the flavourings and volatiles produced. Top and bottom brewing yeasts are classified based on their mode of action. The primary distinction is in the structure of the cell wall. Top yeast cell walls are more hydrophobic; bottom yeasts do not sporulate and adapt to low temperatures, so they are preferred for lager production. Floating brewing yeasts are almost always used to make pilsners and produce strong fermentation at high temperatures (15-22°C). Natural yeast ferments soluble sugars into ethanol, carbon dioxide, and intermediate organic metabolites that have a significant impact on beer flavour and aroma (Bamforth, 2009; Hardwick, 1995; Hough et al., 1993; Priest and Stewart, 2006; Serna-Saldivar, 2010b). Today, yeasts are genetically engineered to produce distinct strains capable of expressing enzymes that influence the brewing process and beer quality.

Major Brewing Operations

Malting

Malting is intended to produce a variety of enzymes necessary for mashing and the remainder for the brewing process. Farming practices, postharvest grain management, and malting conditions all have a significant impact on malt quality. Steeping or soaking, germination, and kilning are

the three major operations in malting. The typical process begins with cleaned barley kernels steeped in cold water (15°C) for an average of 24 hours to activate the grain. The key step is germination of the hydrated seed until the maximum DA is reached, which typically occurs after three to five days, followed by kilning to stop activity and develop important flavouring and colourful compounds. There are various malting technologies, but the most common are floor, drum, rectangular or Saladin, and tower points out the advantages and disadvantages of these commercial malting systems.

Steeping

The goal of steeping is to liquefy the kernel under aerobic conditions in order to activate gibberellic acid synthesis, which controls the germination process. Water enters the kernel through the germ's mycropylar region and rapidly hydrates both the embryonic axis and the scutellum. The water is then distributed throughout the kernel via the endocarp tube cells. Because the endosperm cross cells act as a seal, water diffuses inward. Water initially permeates the aleurone layer before slowly hydrating from the outer to the inner starchy endosperm. Under ideal conditions, the hydration process takes one to two days. The ingestion of the germ causes respiration and the secretion of gibberellins in both the embryo and the scutellum, and the absorbed water catalyses enzyme synthesis and germination, weakening the grain structure. In most cases, the soaking operation is as simple as immersing or spraying water on the grains for 24 to 80 hours to increase grain moisture to 42-48 percent. The majority of operations are carried out in vertical tanks with conic bases or vertical cylinders.Air is injected through perforated pipelines in these containers. The temperature of the water is important because it affects the hydration rate and the microbiology of the grain. A relatively warm water temperature promotes microbial growth, reducing oxygen availability for the developing embryo. The water temperature is typically set at 15°C. Another important control factor is the application of air at regular intervals, which creates aerobic conditions and removes the heat and carbon dioxide produced by respiration. The aeration cycles occur every less than an hour. Soaking is usually interrupted after 12-24 hours by draining the water and then replacing it. The barley kernels absorb water during steeping, which catalyses respiration, enzyme synthesis, and germination.

Germination

The soaked barley kernels are allowed to germinate under special conditions after steeping in order to achieve the desired DA, cell division, and the development of rootlets (primordial roots) and acrospires (first leaf). Typically, the process is carried out on malting beds located inside special rooms with strict temperature and relative humidity controls. This process takes four to six days and is heavily influenced by the temperature of the malting house. The rate of sprouting is determined by the initial moisture, germination temperature, and airflow that circulates through the malting bed.

The process can be carried out on malting floors or in rotative drums. The primary goal of malting is to maximise DA while minimising dry matter loss. Gibberellins are primarily responsible for initiating the synthesis of key enzymes that degrade nutrients stored in the germ's and endosperm's first and second reserve tissues, respectively. The embryo produces two major

hormones, gibberellic acids (GA1 and GA3). These hormones are later transported to the endosperm via the tube cells and induce aleurone cells to synthesise enzymes such as amylases and proteases that catalyse the depolymerization of stored starch and endosperm proteins

The enzymes that are synthesised sequentially during germination. The first enzymes produced during malting are oxidative and reducing, which are associated with oxygen absorption required for respiration, followed by citases, which hydrolyze cell walls and allow other enzymes to enter the endosperm. Phytases, lipolytic enzymes, fibrolitic, proteolitic, and amylolitic enzymes are then produced. Lipases A1 and A2, phospholipases A1 and A2, and lipooxygenases are the most important lipolytic enzymes. Lipases liberate free fatty acids from triglycerides in the germ and aleurone, whereas phospholipases liberate phospholipids. After oxidation, the resulting glycerol is metabolised via glycolysis, and the fatty acids via the -oxidation pathway by sequentially removing acetyl CoA, which enters the TCA cycle for complete oxidation to carbon dioxide and water. The majority of the phosphorus found in cereal grains is in the form of phytic acid (mioinositol hexaphosphate), which is stored in phytic bodies, which are primarily found in aleurone cells. These molecules are responsible for the binding of potassium, magnesium, and other minerals.

These compounds are degraded by phytases, which release phosphate, other minerals, and myoinositol. Myoinositol is a known sugar precursor associated with cell-wall polysaccharides and a seedling growth promoter. Free phosphorus is critical for the synthesis of nucleic acids and lipid membranes for cellular membrane proliferation, as well as the production of ATP and energy. Fibrolitic or cell wall degrading enzymes include cellulases, hemicellulases, pectinases, xylanases, -glucanases, and others that work together to attack cell walls and improve the entry of other enzymes into cells. Because the enzymes are secreted primarily from the aleurone or scutellum, they approach their substrates from outside the cells of the starchy endosperm.

The simple sugars released by wall polysaccharides contribute to the overall energy required for seedling development. Proteolytic enzymes degrade protein fractions associated with the germ (globulins and albumins) and endosperm (prolamins and glutelins) to generate energy and make starch granules more susceptible to amylolytic enzymes. Endopeptidases, carboxypeptidases, aminopeptidases, and peptide hydrolases are enzymes that degrade proteins into small peptides and free amino acids. Endopeptidases degrade proteins that are primarily associated with protein and aleurone bodies, resulting in lower molecular-weight polypeptides and peptides. The primary function of aleurone proteins is to provide amino acids for the synthesis of important enzymes such as amylases. After deamination or keto acid formation, free amino acids are used for enzyme synthesis or oxidised for energy. They hydrolyze conjugated proteins associated with amylases, so helping to activate starch degrading enzymes.

The developing embryo uses free nitrogenous compounds, quantified as free amino nitrogen (FAN), as a substrate. Amylases are the most important enzymes. Most bound -amylases are liberated or activated during germination, whereas -amylases are synthesised. The highest DA is obtained when the malt develops an acrospire that is approximately two-thirds the length of the kernel. Endosperm starch granules are degraded into hydrosoluble maltose and dextrins by - and -amylases. Limit dextrinases or pullulanase and amyloglucosidase further degrade the dextrins to

glucose. Alpha amylase hydrolyzes 1-4 glycosidic bonds of amylose and amylopectin at random, yielding linear and branched dextrins, whereas -amylase cleaves successive maltose units, beginning at the non-reducing end of amylose, amylopectin, and large dextrins. When -amylase comes into contact with a 1-6 glycosidic bond, its catalytic activity stops. Other enzymes, such as starch phosphorylase, cleave glucose units from the non-reducing end of both types of starches by introducing phosphate instead of water, resulting in activated glucose-1-phosphate molecules. -glucosidase hydrolyzes maltose to glucose. When compared to sorghum (Sorghum bicolor) or the other cereals, the germinating barley kernel produces a more favourable and balanced to-amylase ratio. As a result, when compared to barley malt, sorghum malt contains less maltose and more dextrins

Kilning

This drying process serves several functions. The first step is to halt seedling germination and botanical growth in order to obtain a shelf-stable product that recovers enzymatic activity upon rehydration. The dehydration programme reduces the moisture content to the point where it can be stored at room temperature for several months.Kilning conditions are critical for producing malts with varying enzyme activities, colours, and flavours. Lager malts are generally less heavily modified (higher soluble sugars and FAN) than ales, which are kilned to a relatively mild regime. Lager malts develop less colour, resulting in pale, straw, or amber-colored beer. Malts destined for ale production are kilned at a higher temperature, resulting in darker coloured malts. Melanoidins are produced by high temperatures from soluble sugars and amino acids. The higher the kilning temperature, the more complex the flavours that leach into the wort and beer. If the malt is kilned at high temperatures, it is possible to create special dark products with burnt and smoky flavours. This is typical of the colour stouts.

Different methods are used to kiln the green malt. The most common method is to dry the malt bed with air that flows through perforated floors, rotary drums, or vertical dryers. Floor drying entails layering malt for nine to 48 hours of forced air kilning. To avoid enzyme denaturation, the first stages are carried out at temperatures between 50 and 65 degrees Celsius. Temperatures can be raised to 100oC once the moisture content of the malt is reduced to about 10%. The use of a higher temperature programme reduces DA while improving aroma and flavour. The malt is typically dehydrated to 4.5% moisture. The malt is cooled with fresh air after kilning, and the rootlets are stripped or deculmed.

The culms are separated because they have a bitter flavour, contain a lot of nitrogenous compounds, and have a lot of nitrosamines. The culms of sorghum, in particular, are high in cyanogenic compounds. A slow rotating cylindrical screen equipped with a set of beaters that separate the rootlets from the malt or pneumatic equipment that impacts the malt to release the rootlets that are lifted by air aspiration are typical deculming devices. The best quality brewing malts are obtained after at least one month of storage or ageing of the kilned malt. The moisture inside the kernel equilibrates during this phase, and as a result, the malt is roll-milled more efficiently.During the malting process, the barley loses 5-10% of its weight. The main chemical changes that barley undergoes during malting are as follows: assayable starch content decreases

from 64 to about 59%, hemicelluloses decrease from 9 to 7%, reducing sugars increase from 0.2 to 4%, and sucrose decreases from 2 to 5%.

Mashing

Mashing is the process of reactivating enzymes produced during germination in order to convert barley but instead adjunct starches and proteins into soluble compounds. The efficiency of this process has a significant impact on yield and beer properties. Prior to mashing, the malt is coarsely roll-milled to increase the flakes of glumes or husks. It is critical that the glumes continue to stay as intact as possible because they will serve as a more efficient filtering bed during lautering (Fig. 1). The goal of mashing is to convert the starch and proteins used as brewing adjuncts into fermentable carbohydrates/dextrins and soluble nitrogen. Dextrins provide the typical beer body while fermentable carbohydrates and soluble nitrogen are important yeast substrates. Water, usually containing controlled levels of soluble salts, ground malt, and brewing adjuncts are the raw materials used regardless of the mashing method. Supplemental enzymes are added to reactors in some mashing processes. Briggs describes the most prevalent mashing procedures.

Infusion mashing is simply the mixing of malt and brewing adjuncts at 63-67°C for about an hour stand. The resulting mash is then filtered until it is clear. The temperature-programmed mashing takes place in two vessels: a mash mixing vessel and a wort separation device, also known as a lauter tun. The mash is stirred and warmed in the mash mixing vessel using a carefully selected programme designed to allow for optimal enzyme conversion. Typically, the temperature programme begins at 35°C and gradually increases to 50, 65, and 75°C after 30 minutes. The mash is then transferred to the lauter tun to separate the sweet wort from the brewers spent grains. A mash and decoction vessel, as well as a lauter tun, are used in the decocotion mashing. The decoction vessel is used to cook cereal. For enzyme conversion, the grist is mixed with water and heated to 35-40°C.

After 1.5 to two hours, one-third of the mash is transferred to the decoction cooker and heated to 65°C for about 20 minutes to enhance starch liquefaction before being heated to boiling. This material is then pumped back into the stirring mash-mixing vessel, raising the temperature of the combined mash to 52°C for about an hour. Another one-third of the mash is decocted and returned to the mash vessel, where the temperature rises to 65°C. Finally, a third decoction is performed, raising the mash temperature to 76°C. After that, the mash is transferred to the lauter tun. The decoction mashing process is time-consuming.

Because the grist formulations typically contain high amounts of starchy adjuncts, most lagers in the American continent are produced using the double-mashing procedure. To achieve complete starch gelatinization, these starchy materials must be cooked. There are two distinct stages to the mashing. The brewing adjuncts are mixed with water and heated to 35°C in the first stage, known as adjunct mash. Following a 30- to 60-minute stand, the contents are heated to 70°C for about 30 minutes, then to 100°C for 30-45 minutes. Because most cereal starches gelatinize at temperatures above 65°C, the goal is to first hydrate the adjuncts and malt before promoting starch gelatinization and conversion. To denature proteins and inactivate the microbial load and

enzymes, boiling is required. Once the adjunct mashing schedule has been completed, the second mash is prepared by mixing and heating the majority of the malt and water to 35°C. The contents of the two vessels are then combined.

The second phase's goal is to hydrolyze the majority of the starch and proteins. This is best accomplished by programming a gradual temperature increase that begins at 35°C and lasts approximately 30 minutes. The temperature is usually increased by 10-15°C until it reaches 70°C. Proteases are favoured by the sequential temperature increase, followed by - and - amylases (optimum temperatures of 60 and 70°C, respectively). The high temperature of the final mashing stage inhibits most enzyme reactions, reduces viscosity, and improves the wort's fluidity and filtering capacity. Brewing with less DA malts and/or brewing grits less prone to gelatinization may necessitate temperature adjustments, particularly during the second phase of mashing.

Lautering/Sparging

After mashing, the mash is transferred to the lauter tun, where the sweet wort is separated from the spent grains of the brewer. This step is frequently a bottleneck in brewing operations. Poor lautering not only reduces production capacity but can also reduce extract yield. Furthermore, a slow lautering process reduces wort quality, which can lead to issues with beer filtration, flavour, and stability.

Wort viscosity is an important factor influencing lautering. To achieve peak performance, the starch and -glucans must be properly hydrolyzed. The lauter tun is a shallow apparatus with a double mesh floor and rakes that loosen the filtrating bed structure, reducing compaction. The mash contents are allowed to precipitate for 30 minutes to aid in the formation of the husk-rich natural filtration bed. Filtration through the double mesh floor separates the sweet wort. In order to reduce wort turbidity, the wort is typically re-circulated through a bed of spent grains during the first stage of lautering. Filtering is typically performed at 65-70°C, with spent grains being sparged with hot water

Hops (Humulus lupulus) are added to sweet wort to impart the distinct flavour and aroma of European beer. The hops are simply added to the wort to promote the extraction of solubles through boiling and lixiviation for 1.5-2.5 hours (Fig. 1). In general, half to two-thirds of the hops are added at the start of the programme and the rest at the end, with the goal of retaining key volatiles that enhance beer flavour and aroma. The enzymes are inactivated during boiling, the wort darkens due to caramelization reactions, and the hopped wort becomes sterile. Evaporation causes water loss of 4-12% during this thermal treatment. Furthermore, some soluble proteins will bind to tannins and precipitate, reducing haze. Spent hops are removed from the hopped in a large whirlpool with a conical trub collector located in the centre of the apparatus's base. The hopped wort is cooled in a heat exchanger to about 6°C for traditional lagers and as high as 15-20°C for ales, adjusted to a predetermined Plato, and aerated with sterile air to increase oxygen, which is important for yeast growth and budding, particularly during the early stages of fermentation. More proteins become insoluble during cooling and are removed by centrifugation.

Fermentation

In special reactors outfitted with cooling coils or jackets, the wort is fermented into beer. The most commonly used equipment is coil conical and hermetically sealed tanks (150–500 hL capacity). Pitch the wort with 1.5-2.5 g dry yeast or 4.5-7.5 g fresh compressed yeast/L. The yeast reproduces asexually by budding during the first stage of fermentation, increasing the biomass by four to five times and utilising the available oxygen. As a result, the reactor conditions gradually change from aerobic to anaerobic. After 12-24 hours of fermentation, the conditions are thought to become anaerobic. The yeast metabolises fermentable carbohydrates and FAN during this phase, producing ethanol but instead fusel alcohols (isopropanol, amylic, isoamylic, and butanol, respectively). Carbon dioxide is also produced during this stage, as well as intermediate organic products that contribute to the distinctive beer flavour.

Lagers are typically fermented with bottom yeast at 7-15^{o}C for eight to ten days, while ales are fermented at nearly 20°C for three to five days. Lagers are matured in closed tanks at 0°C for four to six weeks to further reduce oxygen to less than 0.2 ppm and enhance bouquet and aroma due to chemical changes such as the formation of diacetyl, dimethylsulfide, and hydrogen sulphide. Yeast cells convert maltose and maltotriose into glucose, which is then metabolised into carbon dioxide, energy, ethanol, organic acids, and volatile compounds. The initial wort density is around 1.040, and the beer density ranges between 1.008 and 1.010 g/cm3. The production of organic acid reduces the pH to 4.2. The change in acidity coagulates some proteins and reduces the solubility of some acidic hop resins even more. Furthermore, during fermentation, significant amounts of FAN are metabolised into fusel alcohols, which affect the organoleptic properties of beer. Beer's sweetness is due to unfermented sugars.Aside from ethanol, fusel alcohols, and hop-derived compounds, many other chemical compounds influence beer flavour, aroma, and stability. A diverse range of esters present in ppm concentrations affect sensory properties. The most important ones are ethyl acetate, ethylacetate, isoamyl acetate, ethyl valerate, isoamyl propionate, phenylethyl acetate, methyl caprate, and methyl and isoamyl caprate, which impart various fruit flavours. Sulfur compounds such as dimethyl sulphide or disulfide, ester or amyl mercaptan, and methional are similarly affected. These sulfur-containing volatiles have an impact on flavor.

Supplementary Enzymes Utilized in Brewing Operations

Malt provides the majority of the enzymes used in brewing. However, a wide range of exogenous enzymes may be added at various stages of the brewing process to speed up production, the ratio of fermentable to unfermentable sugars, and thus the wort fermentability and beer characteristics. Exogenous enzymes are primarily used to enhance extract recovery, the critical operation of lautering or wort filtration, and to improve beer quality, appearance (clarity and haze), and shelf-life. These enzymes are primarily of microbial origin, though some are derived from plants and even animals. These enzymes are commonly supplemented during mashing or wort fermentation in most cases and should be approved for use in foods. Because intrinsic malt and supplementary enzymes have different optimum catalytic conditions, particularly in terms of temperature, pH, and the presence of metal ions, brewers must give them the time and conditions they require to perform their intended task.

α-amylases

These are endoglucanases that catalyse the random cleavage of internal 1-4 glycosidic bonds in starch to produce linear and branched dextrins and oligosaccharides. Hydrolysis is resistant to 1-4 bonds near 1-6 branches. Because of the high reduction in viscosity of the gelatinized starch, the process of converting starch into dextrins (average of 10 glucose units) with -amylase is known as liquefaction. Most -amylases work best at pH levels ranging from 5.5 to 7 and in the presence of calcium ions. The -amylase found in barley and other cereal grains works best at 60°C, whereas most microbial counterparts used in the sweetener and bioethanol industries today are thermostable and work best at temperatures ranging from 85 to 95°C. These thermostable enzymes can withstand temperatures as high as 130°C. The higher conversion rates of these enzymes reduce catalysis time, and the thermal treatment aimed at starch gelatinization can be integrated into liquefaction. The disadvantage, however, is the difficulty in inactivating the enzymes to prevent further undesirable catalysis.

Amylases are very effective at liquefying starch and work in tandem with counterparts found in barley malt or are used to enhance the action of low DA barley malts. In the industry, both fungal and bacterial -amylases are commonly used. Fungal -amylases are typically derived from Aspergillus oryzae, whereas heatstable bacterial -amylases are typically derived from a genetically modified strain of the genus, such as *B. subtilis* or Bacillus amyloliquefaciens (Table 2). Bacillus stearothermophilus and *Bacillus subtilis* are two other microorganisms that have been identified. Aspergillus enzymes have optimal pH and temperature ranges of 5.0 to 6.5 and 55 to 65°C, respectively. Alpha-amylase extracted from *Bacillus subtilis*, on the other hand, has an optimal temperature and pH range of 65 to 70°C and 6.0 to 7.5.

At temperatures below 70°C, the heat-stable amylase from *Bacillus licheniformis* is ineffective and should be used to thin or hydrolyze the starch as it gelatinizes. When added to the colder malt mash, this prevents high wort viscosity and minimises starch retrogradation. One of the primary benefits of supplemental -amylase is that it allows for the use of more adjunct in the cooking vessel while also preventing the formation of off flavour from the husk of the barley malt. Despite the very complete digestion of starch, hydrolysis with -amylase yields very few fermentable carbohydrates. Adequate-amylase exercise during mashing is unquestionably necessary for acceptable wort runoff during mash filtration. Malt usually has more than enough -amylase to allow for good runoff. If there is a problem with starch degradation, Bacillus amyloliquefaciens bacterial amylase has been proposed to improve runoff. Aspergillus oryzae alpha-amylases have also been used to prevent starchy haze caused by high-molecular-weight dextrins. When used at a high concentration, this enzyme will increase the amount of fermentable sugars during fermentation.At lower concentrations, however, they breakdown dextrins without significantly increasing fermentable sugar levels. Even at cold storage temperatures, preparation of this enzyme effectively reduces starchy hazes that form in beer during storage within a day.

CHAPTER 6

BASICS OF B-AMYLASES (1-4 D GLUCAN MALTOHYDROLASES)

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Beta-amylases (1-4 D glucan maltohydrolases) are alone as found only in plants and bacteria that convert linear and branched dextrins from the non-reducing end into maltose and lower molecular-weight dextrins.When a -1-6 glycosidic bond is reached, the -amylase activity stops. It is known as the complementary amylase because it absorbs water dextrins produced by -amylase. Most -amylases have optimal pH and temperature ranges of 5.0-5.5 and 55-60o C, respectively.Supplemental -amylases can be extracted from specific bacterial strains such as Bacillus polymixa or Bacillus cerus. -amylases are primarily used to increase the amount of fermentable maltose and thus the fermentability of wort. They can also be used to reduce dextrins and the caloric content of light beers.

Their optimum pH and temperature are 5.3 and 55°C, respectively; thus, they work well at the typical acidity of mashes. Heat easily denatures beta-amylases, which are completely deactivated before lautering. In mashing, Norris and Lewis (1985) used food-grade commercial barley-amylase. The wort fermentability was affected beyond the extract yield when the mash temperature was varied between 60 and 80°C and the adjunct ratio was varied between 0 and 98 percent. The added enzyme significantly increased fermentability and could be useful in the production of low-dextrin or light beers.

Limit Dextrinases/Pullulanases

Limit dextrinases, also known as pullulanases (dextrin endo 1-6 glucosidase), are debranching enzymes that catalyse 1-6 glycosidic linkages but not 1-4 glycosidic bonds (Bryce, 2003). These enzymes are thought to be complementary to amylases in terms of increasing starch conversions into fermentable sugars, primarily in terms of maltose concentration (Table 2, MacGregor, 2003). MacGregor *et al.* modelled the contributions of -amylase, -amylase, and limit dextrinase to starch degradation during mashing and concluded that adding limit dextrinase to the mashes resulted in a significant increase in fermentable carbohydrates levels. When combined with high levels of -amylase, the limit dextrinase had a synergistic effect on increasing maltose levels in the mash liquor.

Today, regular and heat-stable pullulanases are manufactured. Regular bacterial pullulanases are typically derived from Klebsiella aerogens, Klebsiella pneumoniae, or Bacillus acidopullylyticus, or from the fungus Trichoderma. These enzymes are most active at pH levels of 4 to 7.5 and temperatures of 50 to 65°C, respectively, and are easily inactivated by heat or beer pasteurisation. Heat-stable pullulanases capable of working at temperatures of up to 90°C can be extracted from strains of Clostridium thermohydrosulfuricum.

Amyloglucosidases

Molds, such as Aspergillus spp. or Rhizopus spp., are commonly used to extract glucoamylases or saccharifying enzymes. Amyloglucosidases attack the nonreducing ends of starch chains and glucose units that release dextrin. Because their hydrolysis on 1-4 bonds is faster than the attack on 1-6 bonds, the addition of limit dextrinases speeds up the conversion of starch into glucose. The optimal pH range is 4.0 to 5.5, and the enzyme can work for extended periods at temperatures ranging from 60 to 65°C. Recombinant yeasts capable of expressing glucoamylase have been developed successfully. Bui *et al.* cloned the Arxula adeninivorans glucoamylase gene in *Saccharomyces cerevisiae*. The transformants secreted 95% of the enzyme into the culture medium, and the glucoamylase activities observed indicated that the soluble sugars and alcohol content would improve after fermentation.Amyloglucosidases are added to mashes, particularly those containing a high proportion of adjuncts, to increase wort fermentability and have proven effective in the production of lager beer with lower viscosity and fewer calories, as well as sorghum beer. The amyloglucosidase enzyme is deactivated at the end of the mashing process or during hop boiling.

Sorghum has recently been identified as a source of brewing adjuncts and diastatic malt for the production of gluten-free beer. When brewing with sorghum, the main issues are the lower DA of its malt, which is especially deficient in -amylase activity, and the comparatively higher gelatinization temperature of its starch when compared to barley starch. In comparison to barley malt, sorghum malt requires modified mashing to increase starch solubility and hydrolysis. Adding extrinsic -amylase or microbial glucoamylase during sorghum wort mashing increased extract and starch conversion and favoured the ratio of fermentable sugars to dextrins. As a result, the best wort composition increased the alcohol content and yield. As a result, the best technology for producing gluten-free sorghum beer is to use white sorghum genotypes suitable for the production of malt and refined grits supplemented with glucoamylase during mashing.

Proteases

In malt and starch-brewing adjuncts, protein is the second most abundant component. 6These raw cereal-based materials contain 10% protein, which is broken down into albumins, globulins, prolamins, and glutelins. The last two are associated with the starchy endosperm and constitute the majority of the fractions. The malting process promotes the formation of proteases such as endopeptidases and carboxipeptidases which break down prolamins and glutelin-yielding peptides during mashing and are commonly measured with the FAN ninhydrin colorimetric assay. Because the water-soluble FAN compounds are an important substrate for yeast during fermentation, a lack of FAN in the wort limits yeast activity and fermentation, as well as the production of acceptable beer.

To break down gluten proteins that impede starch hydrolysis during mashing, various supplementary proteases have been used. This is especially true when sorghum malt or refined sorghum adjuncts are used. Because of the strong interaction between protein and starch granules and sorghum malt's lower protease activity, worts made from sorghum malt and grits are typically low in FAN compounds. As a result, plant-derived (ficin, papain, and bromelin) and

microbe-derived proteases improve wort extraction and assayable FAN. Proteins are hydrolyzed by these enzymes at temperatures as high as 65°C. According to Ryder and Power (2006), the production of too many soluble nitrogenous compounds can reduce foam formation and foam stability.

Additionally, proteases are used to degrade polypeptide haze precursors in beer. Plant proteases derived from papaya (Carica papaya), pineapple (Ananas spp.), and figs (Ficus spp.) have all been successfully added to beer as stabilising agents. These proteases degrade the haze precursor in a similar manner and are denatured by thermal treatment or pasteurisation. To prevent haze during cold storage, papain was also added after the yeast was removed from the beer.Microbial proteases have an advantage over plant proteases in terms of degrading foam precursors. *Bacillus subtilis* proteases function well at temperatures ranging from 45 to 50°C and mash pH values of around 5.6. Fungal proteases have an optimum pH of 3 to 6 and a temperature of around 50°C.

Beer stability has been improved by using a proline endopeptidase extracted from Aspergillus niger. Because of its increased proline content, this enzyme specifically hydrolyzes sensitive polypeptides and promotes beer physical stability while having no effect on foam stability. Lopez and Edens (2005) used a prolinespecific protease to hydrolyze proteins that promote haze in beer wort. The pre-digestion of proline-rich wheat gliadin with various proteases revealed a strong haze-suppressing effect by this specific enzyme. This discovery was confirmed in small-scale brewing experiments with a proline-specific protease that can hydrolyze at acidic pH. Subsequent pilot plant trials demonstrated that even low levels of this enzyme, when added during the fermentation phase of beer brewing, effectively prevented chill-haze formation in bottled beer.

Recent research has also suggested that prolyl endopeptidases be used to degrade gluten in foods and beverages. These enzymes are known to be found in germinated cereal grains, bacteria, and fungi. Aspergillus niger is the most common source of prolyl endoproteinase. Guerdrum and Bamforth investigated when the gluten of barley was hydrolyzed when prolyl endoproteinase was supplemented in an attempt to produce gluten-free beer. During mashing and lautering, approximately half of the prolamin was lost, most likely due to proteolysis or precipitation. Only 1.9% of the original hordein from the malt remained in the unfiltered beer after fermentation. These authors concluded that the group had significantly in the beer after fermentation represented less than 2% of the total prolamin in the malt, rendering the beer gluten-free and without reducing foaming capacity.

Cellulases and β-glucanases

Cellulases are -glucan hydrolyses that attack the 1-4 linkages of cellulose (Tenkanen et al., 2003). Cellulases are not the most common enzymes used in the brewing industry, but they are used to enhance the activity of other, more important enzymes such as -glucanases and xylanases. Cellulase preparations from Penicillium funiculosum or Penicillium emersonii were added to the barley mash to break down the -glucans, holocellulosic material, and pentosans. The first enzyme cocktail demonstrated adequate activity inside this pH range of 4.3 to 5.0 and temperatures ranging from 65°C to 80°C, while the second demonstrated adequate activity at

temperatures ranging from 65°C to 80°C.As cell-wall components, -glucans contribute a minor portion of cereal weight but have a significant impact on processing and nutritional value due to their proclivity to be extracted with water and then form viscous solutions (Hramova and Fincher, 2003). These soluble fibre components are primarily composed of long, linear chains of glucosyl residues linked by 1-3 and 1-4 bonds. It is well known that undegraded wort-glucans have a negative impact on beer filtration, reducing filter capacity and increasing the consumption of filter aids such as diatomaceous earth, which is used to remove beer haze.

This enzyme works in a similar manner to barley glucanase, resulting in a rapid size reduction with virtually no production of fermentable sugars. Various fungal -glucanases (Aspergillus and Trichoderma) have been used successfully to break down -glucans during mashing. These enzymes perform best at pH levels between 4 and 5. The fungal enzymes derived from Aspergillus niger and Trichoderma reesei work effectively at beer pH 4.2 and exhibit good activity at temperatures above 60°C. Trichoderma glucanase is particularly effective at improving beer filtration.

Silveira Celestino *et al.* (2006) investigated the use of exogenous Rhizopus microsporus -glucanases to reduce the -glucans already present in barley malt.The optimal pH and temperature ranges for hydrolysis of 1,3 and 1,4-glucans were 4-5 and 50-60°C, respectively. The purified enzyme was able to reduce mash viscosity as well as filtration time or runoff. The relative viscosity value for the mash treated with fungal 1,3-1,4-glucanase was consistently lower than the values determined for the mash treated with commercial glucanases. During storage, the same -glucanases can be added to beer. However, because these enzymes have a relatively high pH optimum, they are less effective in beer than in the mash.

Xylanases

Hemicellulose is made up of glucans, mannans, arabinans, galactans, and xylans as well as a matrix and cross-linking heteropolysaccharides. Gluco and arabinoxylans are hydrosoluble chemicals found in cereal cell walls such as maize, sorghum, wheat, rye, and triticale. Many bacteria, fungi, yeast, and protozoa degrade these compounds. During germination, xylanases are synthesised and catalyse the hydrolysis of hemicellulose, removing the physical barrier imposed by cell walls on the free diffusion of amylases and proteases. Brewers use xylanases, like -glucanases, to improve wort separation, speed up beer filtration, and reduce beer haze. These preparations may contain complex enzyme mixtures that work synergistically.

Du *et al.* recently identified three novel thermophilic xylanases from Humicola insolens with brewing potential. Recombinant XynA-C produced in Pichia pastoris demonstrated optimal activities at pH 6.0-7.0 and temperatures of 70-80°C, as well as good stability across a wide pH range. The enzyme properties of the gene xyn C produced by Humicola insolens were similar to those of the one expressed by Pichia. XynA demonstrated improved alkaline adaptation and thermostability, as well as increased catalytic efficiency and substrate specificity. Under simulated mashing conditions, the addition of XynA-C outperformed a commercial enzyme used by brewers in terms of filtration acceleration (37.3%) and viscosity reduction (13.5%).

Many other enzymes with various functions have been proposed for use in brewing operations. These enzymes include tanninases, phosphatases, oxidases, transglycosylases, and glucosidases. These are used as processing aids or to degrade metabolites that have a negative impact on the flavour and aroma of beer

Acetoacetate Decarboxylase

It is critical to manage diacetyl and other vicinal diketones (VDK) during beer fermentation and maturation because these compounds affect beer taste and shelf-life. Brewers have used -acetolactate decarboxylase to decarboxylate -acetolactate into flavor-inactive acetoin, preventing diacetyl formation.Thus, this decarboxylase has been commercially used to remove -acetolactate and -aceto—hydroxybutyrate to levels below the volatile VDK taste threshold without affecting other important beer features Commercial enzymes that are generally regarded as safe are typically derived from Acetobacter aceti, which is typically added to sweet wort prior to pitching or fermentation. Godtfredsen and Otresen (1982) investigated beer maturation using Enterobacter aerogenes-derived -acetolactate decarboxylase. To prevent off flavour development, the enzyme was applied at 10°C during beer maturation. When compared to conventionally prepared beer, beer matured in the presence of the enzyme was judged to be of equal quality successfully cloned a gene from Acetobacter aceti ssp. encoding -acetolactate decarboxylase into brewer's yeast..The transformed yeast grown at the laboratory scale significantly reduced the total diacetyl concentration, as expected.

Enzymes in Baking

Breadmaking is based on several fundamental complex physical, chemical, and biochemical changes such as evaporation of water, volume expansion and formation of a porous structure, denaturation of nutrient, gelatinization of starch, crust formation, and browning reaction, all of which are necessary for the final product's quality. Bread has been one of the primary forms of food since ancient times and is a major food product all over the world. Wheat bread is the most popular and widely consumed type of bread, and it is a good source of nutrients.

Bread, in particular, provides complex carbohydrates, dietary fibre, a low fat content (no cholesterol), minerals, particularly calcium, phosphorus, iron, and potassium, and B vitamins. A number of European countries recommend a daily bread consumption of approximately 250 g, which corresponds to four to eight slices, depending on national food habits (WHO, 2003). The main ingredient in baked goods is flour made from cereal grains, which are the fruits of grass family plants (Gramineae). Aside from wheat, other major cereals used in bread production include corn, rice, barley, sorghum, millet, oats, and rye. They are grown on approximately 60% of the world's cultivated land. Wheat, corn, and rice account for 90% of cereal grain production worldwide, the most cereal grains (FAO, 2012), and provide two-thirds of human energy requirements through diet (Bharath Kumar and Prabhasankar, 2014). Different types of grains have an impact on the final product's quality and nutritional value. Consumers' perceptions of bread quality in terms of health have recently improved.

Consumption of whole grain foods and high dietary fibre, including bread, has been linked to the prevention of metabolic syndrome, obesity, and associated chronic diseases such as

cardiovascular disease and Type 2 diabetes. Bread has evolved into a good vehicle for incorporating a good supply of dietary fibre, a variety of vitamins and minerals, and a source of energy in the form of starch. Furthermore, total bread consumption has increased over the last 20 years. Bread and bakery goods are widely consumed by the global population due to their low cost and high nutritional value. Because of consumer demand, the bakery industry is becoming increasingly active in terms of processing, recipes, shapes, and the production, wholesale, and retailing of baked goods such as breads, cakes, pastries, breakfast cereals, snacks, cookies, and crackers. Nowadays, the bakery industry benefits from optimising bread quality in terms of primary ingredients, advanced technology design to monitor the process using approved methods, but also from identifying and incorporating values that are appealing to different consumer segments. In the baking industry, research has primarily focused on investigating methods to improve the finished version by extending shelf-life and preserving product freshness.

Enzyme research has played an important role in cereal processing, particularly in the baking industry, as they serve as processing or technological aids. Another advantage is that enzymes are considered clean label compounds. Chemical supplements used in the baking industry are gradually being replaced by enzymes, which are the best and safest alternative due to their GRAS (Generally Recognized as Safe) status. Enzymes can catalyse chemical reactions but are rendered inactive after breadmaking due to denaturation of their protein structure during baking. Baking enzymes, which can modify and improve the functional, nutritional, and sensorial properties of ingredients and products, have become an essential part of the industry. As a result, enzymes have a wide range of applications in the processing and production of bread products. Traditionally, enzymes were used as processing aids in the baking industry, but due to increased nutrition awareness, a broader concept has been adopted, and they are now regarded as healthy aids due to their role in improving the nutritional quality of bakery products.

Enzymes that contribute to the quality and processing characteristics of bread can be derived from endogenous enzymes found in grain raw materials or commercial enzymes of microbial origin that are added during the breadmaking process or supplemented with flour. There are a variety of commercial enzyme preparations and enzyme-containing bread improvers available. To achieve the desired functionality, many enzymes are used in industrial baking (Rosell and Collar, 2008; Rosell and Dura, 2015). Enzymes are classified based on the reactions they catalyse and the substrate on which they act. The International Union of Biochemistry and Molecular Biology's Nomenclature Committee assigns an EC number to each enzyme (Cornish-Bowden, 2014). Amylases, proteases, lipases, hemicellulases, and oxidoreductases are the most commonly used enzymes in the baking industry. All of these enzymes, when used individually or in combination, improve dough performance and stability, dough strengthening and bleaching, bread volume, crumb softness, crust colouring or browning, and anti-staling properties. Their action during breadmaking is heavily reliant on the flour constituents, so it is critical to understand them in order to understand the enzymes' functionality in baking.Rapid advances in biotechnology have enabled improved fermentation yields, modification of enzyme specificity, selectivity, and stability, and an increase in the number of enzymes available to the baking industry.

Principles of Breadmaking

Cereals have played an important role in human nutrition since ancient times, particularly common wheat, which is most commonly used for bread and baking goods. As previously stated, many other grains, such as millet, quinoa, sorghum, oat, barley, rye, and teff, have been used to meet customer needs or special nutritional needs.

Cereal grains contain 66-76 percent carbohydrates, with starch being the most abundant carbohydrate and the most important reserve congress person of cereals (55-70 percent), followed by minor constituents such as arabinoxylans (1.5-8%), -glucans (0.5-7%), sugars (3%), cellulose (2.5%), and glucofructans (1%). Proteins are another important group of constituents, accounting for approximately 8-11% of total. With the exception of oats (7%), cereal lipids (2-4%) are minor constituents, along with minerals (1-3%). Bread is made by baking a mixture of flour, which is the most important ingredient and a key source of enzyme substrates, water, salt, and yeast.

Breadmaking is a traditional skill that is practised all over the world. Regardless of the variety of cereal grains and processed bread products, a number of central stages are shared by all bread products and breadmaking processes. Breadmaking fundamentals include ingredient mixing, dough fermentation, rounding and moulding of dough, proofing, cooking of bread in an oven, and cooling. The goal of the breadmaking process is to convert bread into aerate, edible, and tasty bread for the consumer through a series of stages that can proceed in different ways depending on the final product but all serve the same purpose.

Mixing Ingredients

The first stage in forming a homogeneous dough is the mixing of flour (primarily wheat), water, yeast, salt, and other listed ingredients in appropriate ratios. This has a direct effect on the performance of the end product. During mixing, fermenting, and baking, the dough undergoes various physio-chemical and biological transformations such as water evaporation, starch gelatinization, volume expansion, crust formation, protein denaturation, browning reactions, and so on, which are heavily influenced by temperature and water content (Rosell and Collar, 2009). Mixing incorporates air bubbles into the dough and provides the necessary mechanical energy for the development of the protein network. The real purpose of vigorous and continuous mixing and kneading is to increase the volume and softness of the crumb. This process alters the physical properties of the dough, making it extensible enough even to expand during proofing and elastic enough to improve its ability to retain the carbon dioxide gas produced by yeast fermentation, as well as stable enough to retain its shape and cell structure

Dough Fermentation

Dough is a macroscopically homogeneous four mixture of starch, protein, fat, salt, yeast, and other ingredients that combine to form a visco-elastic material with rheological properties intermediate between a viscous liquid and anelastic solid.Dough development begins with the mixing of ingredients and the formation of gluten, which requires the hydration of flour proteins as well as the application of energy via the kneading process. Gluten proteins (gliadin and

glutenin) influence wheat baking quality by imparting water absorption capacity, cohesivity, viscosity, and elasticity to dough. Gliadins are known to have viscous properties, whereas glutenins have strength and elasticity (Shewry et al., 1986). Gluten rheological properties are a direct result of wheat quality and affect the textural characteristics of the finished bread. As the primary carbohydrate in flour, starch can form a continuous network of particles with the macromolecular network of hydrated gluten, influencing the rheological properties of doughs other constituents, such as non-starch polysaccharides and lipids, can influence dough stability and thus breadmaking performance.

Mixing, yeast metabolism results in carbon dioxide production and dough expansion only if the carbon dioxide gas is retained in the dough during fermentation. Fermentation occurs when air bubbles that were previously incorporated during mixing expand, resulting in the characteristic aerated structure of bread. During alcoholic fermentation, enzymes present in yeast and flour or added intentionally break down starch and oligosaccharides into carbon dioxide and alcohol. The expansion of the dough, and thus the final volume and texture of the baked product, is determined by the growth of gas bubbles.

Proving and Baking

Proving refers to the final rise that the dough experiences during a resting period after it has been shaped but before it is baked. After the dough has been proofed, baking occurs through the stabilisation of a porous structure by changing the molecular configuration of the polymeric components in the cell walls using heat. Thermal transitions occur at bread baking temperatures and moisture contents. Baking temperatures may vary depending on the oven and the product. Baking time, oven temperature, and heat source all influence bread quality during the baking process. The initial temperature raises the volume of the dough, causing rapid evaporation of water and the release of carbon dioxide, ethanol, and some aromatic compounds. Structure changes also occur during the bread-baking process, which promotes starch gelatinization and protein denaturation with the coagulation of gluten, which includes solidification and expansion. The rise in temperature and lower moisture content cause a non-enzymatic browning reaction, followed by crumb development and, finally, gradual colour development.Because of the inherent variability of baked goods, the breadmaking process can differ from one product to the next. In breadmaking, replacing wheat flour with significant amounts of other cereal or sort of anti flours can change the process and produce a suitable product.

Enzymes Role in Breadmaking to Improve Process

Proteins that catalyse chemical reactions and convert substrates into different molecules are known as enzymes. Enzymes are heat-sensitive biological catalysts with an optimum temperature and pH for activity. Their activity is also affected by the availability of water, the amount of enzyme used, the availability of substrate, and the time allowed for the reaction. Enzymes reach a denaturation point and lose functionality once the optimal temperature has been reached its maximum tolerance. Baking enzymes have been an important part of the industry for centuries because they can modify and improve the functional, nutritional, and sensorial properties of ingredients and products; as a result, enzymes have found extensive applications in the

processing and production of bread products. Enzymes (α-amylases, xylanases, lipoxygenases, glucooxidases, transglutaminases, proteolytic enzymes and lipases) are added to improve the dough-handling properties, loaf volume, fresh bread stability and shelf-life of bread but also lipoxygenase used for dough strengthening and bleaching, hydrolases such as amylases.to convert starch to sugar and produce dextrins), proteases and hemicellulases which affect wheat gluten, and lipases which improve the dough's rheological properties and baked product quality. All of these enzymes are important in maintaining bread volume, crumb softness, crust crispiness, crust colouring or browning, and freshness.

Nowadays, the baking industry is responding to rising consumer expectations by claiming more natural products and new products tailored to specific nutritional needs. Enzymes have a number of advantages in this tendency, including being used as an alternative to traditional chemical-based technology, lowering energy consumption levels, and producing fewer by-products, and not requiring any changes in the processing line operation during the breadmaking process.

As a result of years of research and development, numerous new methods for modifying enzymes or increasing their yield have been developed, making their use economically viable in breadmaking. The most common way to use those enzymes is to combine them with the rest of the ingredients during the first stage of breadmaking. When adding enzymes, there are no special requirements, making them extremely useful as processing aids. Lipases can be added individually or in fibreglass mixtures, which can act synergistically, additively, or antagonistically in the bread-making process. It is common practise to supplement flour and dough with enzyme improvers to achieve the desired effects, provide consistent product quality, or increase yield in the process, and their action will continue throughout all stages of breadmaking. As a result, this section focuses on the enzymes used as processing aids in the breadmaking process, whether they are added as a single enzyme or in combination.

CHAPTER 7

CLASSIFICATION OF THE AMYLASES

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Amylases are a group of amylolytic enzymes that are capable of breaking down glycosidic linkages in starch to form smaller dextrins, which are then fermented by yeast. Enzymes such as malt and fungal -amylase have long been used in breadmaking. It is critical to remember that the source of enzymes determines their activity and stability F ungal enzymes are generally less thermostable, and the majority of their activity is lost during starch gelatinization. The addition of amylases is primarily intended to improve the amylase activity of the flour (flour standardization). The fermentation rate is increased by enzyme supplementation of flour due to an increase in fermentable sugars.

The viscosity of the dough is reduced, which improves the volume and texture of the product. It also produces more sugars in the dough, which improves the bread's flavour, crust colour, and toasting qualities. The action of -amylases is visible in the crumb microstructure, which has been linked to structural changes promoted at the dough level. Initially, the effect of -amylases was attributed to increased sugar content, but it has since been confirmed that the effects extend beyond sugar production. Patel *et al.* investigated the effects of fungal -amylase on chemically leavened doughs and discovered that the baking qualities of chemically leavened dough improved when fungal -amylases were present. The higher amount of free liquid and lower viscosity in the dough containing -amylases were attributed to differences in rheological properties. Furthermore, Sahnoun *et al.* discovered that adding a preparation containing an adequate concentration of -amylase from Aspergillus oryzae improves dough properties and bread quality by increasing dough resistance to extension and bread volume. It should be noted that some bacterial -amylases have high thermostability and may survive baking.

As a result, they are difficult to control during storage and can result in gradually decreasing crumb structure properties. According to Bosmans et al., adding three different bacterial amylases to a bread recipe affects bread firming, resulting in lower initial crumb resilience. Bread loaves with Bacillus stearothermophilus -amylase retained the best quality, whereas *Bacillus subtilis* -amylase caused extensive degradation of the starch network, resulting in partial structure collapse and poor crumb resilience.Fresh bread has a relatively short shelf life when stored due to a number of complex physicochemical changes known as staling, which include an increase in crumb firmness. The retrogradation of the starch fraction in bread is critical to staling. After the initial cooling process, retrogradation/recrystallization of starch, specifically the short amylopectin side chains, plays a significant role in bread firming. To a large extent, the quality of bread products is defined by taste and freshness. It is of significant economic importance to the bakery industry because it reduces the shelf-life of baked goods. Several additives and

different combinations have been used to delay staling and improve bakery product texture, volume, and flavour. Enzymes active on starch, primarily amylases, have been proposed to act as anti-staling agents, with maltogenic -amylase being a particularly effective anti-staling agent.

Armero and Collar proposed a combination of emulsifiers, hydrocolloids, and -amylase for retarding staling in white and wholemeal breads, with different synergistic and antagonistic effects (DATEM*SSL, -amylase*SSL, -amylase*HPMC). Amylases have been shown to have anti-firming properties in bread staling by numerous authors. Gomes-Ruffi *et al.* investigated the effect of maltogenic amylase in combination with an emulsifier on the quality of pan bread during storage, resulting in an increase in bread volume, a decrease in firmness, and high sensory scores with multiple combinations of the emulsifier and the enzyme.

Although enzymes are typically added during the mixing process, the use of enzymes on the dough or even on the surface of partially baked breads has been reported. Primo-Martin *et al.* investigated the effect of different enzymes (endoprotease, transglutaminase, and -amylase) sprayed on the dough surface as a possible strategy for extending crust crispiness, finding that protease had the greatest effect. Amyloglucosidase sprayed on the partially baked loaf surface, on the other hand, has been proposed for modulating the properties of the bread crust and increasing its crispness by increasing the number of voids and texture fragility

Non-starch Hydrolases

Xylanases from families 8 and 11 were found to be very effective as baking processing aids, with Aspergillus oryzae xylanase being the best bread improver and Trichoderma signal xylanase being the best anti-staling improver. Ahmad *et al.* investigated the effect of Aspergillus niger xylanase on wheat kernel tempering and dough mixing. Both treatments altered the dough's properties, improving dough performance and resulting in a larger loaf volume and better bread sensory characteristics. However, the overall development was more pronounced in the tempering samples that received enzyme treatment.

The hydrolytic activity of xylanases resulted in monomers and oligomers that affect the water balance, transforming protein-starch interaction, and thus improving dough machinability, dough stability, oven spring, loaf volume, crumb structure, and shelf-life, as well as lowering the staling rate. McPhillips *et al.* investigated the purification of an extracellular endo-1,4-xylanase and its application in breadmaking (2014). The enzyme's optimal activity was measured at pH 6.0 and 65°C, which was suitable for breadmaking applications. The hydrolysis products of the enzyme's action on 1,4--Dxylosidic linkages resulted in xylosaccharides, primarily xylotriose and xylobiose.

At low dosages, the basic wheat bread recipe demonstrated its ability to increase loaf volume and softness while reducing bread staling by up to four days of storage. The arabinoxylans (AX), the majority of non-starch polysaccharide (85-90%), have a backbone of -(1,4)-linked xylose residues. They exist in two forms: water-extractable (WE-AX) and moisture (WU-AX). Their structure results in unique physicochemical properties that heavily influence their functionality in breadmaking. Dornez *et al.* investigated the individual activities of three psychrophilic xylanases and one mesophilic xylanase.

Xylanases with a high capacity to solubilize WU-AX during mixing increased bread volume more than xylanases that solubilized WU-AX primarily during fermentation. The efficiency of xylanases could be related to their substrate hydrolysis behaviour and was primarily determined by the enzyme's temperature-activity profile and inhibition sensitivity. Xylanases have also been found to be useful in various types of flour two organic spelt cultivars with different dough rheological properties was also investigated. For weaker spelt cultivars, the optimised formulations allowed for a significant increase in specific volume and crumb softening.

Pentosans in flour play an important role in bread quality because of their ability to absorb water and interact with gluten, which is required for the formation of the loaf structure. Because of their high elasticity and low extensibility,'short' gluten flours cause problems in breadmaking by reducing the dough's ability to retain gases released during fermentation. Stoica *et al.* found that combining a reducing agent (L-cysteine) and a fungal pentosanase increased bread volume, porosity, and elasticity.Anti-staling effects of other enzymes acting on non-starch cereal carbohydrates have been investigated. The simultaneous analysis of hardening and starch retrogradation revealed that the anti-staling effect of xylanase could be attributed to the delay in starch retrogradation, which had the greatest anti-staling effect. In contrast, cellulose and -glucanase slowed bread firming, but another mechanism must be at work. The use of enzymes such as xylanases has been shown to play a significant role in increasing bread shelf life and re6ducing bread staling.

Tranglutaminase (TGase)

TGase is a protein-glutamine -glutamyl-transferase that catalyses an acyl-transfer reaction between the -carboxyamide group of peptide-bound glutamine residues and a wide range of primary amines. TGase has been used in breadmaking to reduce dough extensibility and increase resistance to stretching due to TGase-induced protein network reinforcement. The use of enzyme improves dough stability and bread volume while also improving crumb structure. This enzyme has also been used to improve the baking quality of weak wheat flours and bug-damaged flours. TGase has also been shown to have beneficial effects on non-wheat bread. Beck *et al.* (2011) demonstrated that an optimal TGase dosage improves the rheological properties of rye dough by forming a continuous protein network, as well as having a positive effect on loaf volume and crumb texture of rye bread. Higher levels of the enzyme were also found to have a negative impact on loaf volume, resulting in an increase in crumb springiness and hardness. Gluten-free bakery products have been one of the research community's most pressing concerns, owing to the growing demand for these products.

The increasing demand for gluten-free products encourages the use of ingredients such as rice flour, corn flour and meal, ancient grains, tubers, and pulses. However, due to a lack of viscoelastic properties, the proteins in those flours are unable to retain carbon dioxide during fermentation, necessitating the search for technological alternatives to mimic gluten viscoelastic properties. However, the protein network formed by TGase is highly dependent on the protein's origin, thermal compatibility, and enzyme dosage. In this regard, TGase has been the most widely proposed enzyme for creating protein crosslinks in rice flour. The authors investigated the addition of increasing amounts of TGase (0.5, 1.0, or 1.5 % w/w) to rice flour to obtain a

progressive increase in the viscous and elastic moduli, but higher bread volume and softer crumb were obtained with 1.0 % TGase, and further improvement was obtained with the addition of 2% HPMC. Furthermore, Gujral and Rosell (2004a) used TGase to promote protein interactions in rice flour, which resulted in improved breadmaking performance. Renzetti *et al.* tested the network forming potential of flours from six gluten-free cereals used in breadmaking (brown rice, buckwheat, corn, oat, sorghum, and teff). The formation of protein complexes by TGase was confirmed using three-dimensional confocal laser scanning micrographs. Better fundamental rheological analysis and bread quality revealed that 10U TGase improved the buckwheat and brown rice batters and breads, but had a trimental effect on corn infielders with increased specific volume and decreased crumb hardness and chewiness on corn breads. A comprehensive review of the role of enzymes in gluten-free flours was recently published. Recently, some proteins (albumin, casein, pea protein, and so on) have been added to boost transglutaminase activity in order to create a protein network in gluten-free matrixes.

Oxidase

Different oxidases have a positive effect on dough development and quality and are mostly attributed to the reinforcement or bolstering effect of dough (Oort, 1996), but they can also be used as dough-bleaching agents. Zhang *et al.* investigated the effect of adding recombinant lipoxygenase to wheat flour whiteness, finding that the enzyme-treated flour had increased volume and loaf height, as well as improved crumb colour, resilience, chewiness, and hardiness. The addition of glucose oxidase (GOX) to dough increases the gluten macropolymer content via disulphide and non-disulphide crosslinking, resulting in protein aggregates and broken segments. A study from the macroscopic to the molecular level conducted by Bonet *et al.* (2006) revealed a reinforcement or strengthening of wheat dough and an improvement in bread quality, though excessive addition of GOX produced excessive cross-linking in the gluten network and was responsible for the negative effect on breadmaking properties. Degrand *et al.* (2015) recently reported the potential value of a carbohydrate oxidase similar to Aspergillus niger GOX as a dough and bread improver. Carbohydrate oxidase has the same efficiency with maltose as GOX does with glucose, regardless of the oxygen supply. GOX has also been used to replace chemical oxidants in the breadmaking process when using weak flours, reducing economic losses caused by plagues. This enzyme was able to repair broken covalent bonds between glutenin subunits while also forming dityrosine crosslinks between wheat proteins, reinforcing the gluten network and providing dough functionality. The use of gluten-free flour, such as rice flour, complicates the breadmaking process. Gujral and Rosell validated the effects of GOX on rice-flour dough rheology and nutrient modification, resulting in rice bread with improved specific volume and texture.

Lipases

Despite the fact that lipids are minor constituents of flour, its use of lipases in breadmaking has been reported as an individual effect. Lipase is primarily used to enhance the flavour of bakery products by esterifying short-chain fatty acids. This enzyme also helps to improve the rheological properties, texture, and softness of the dough. Lipase, when used as a dough and bread improver, reduces crumb pore diameter, increases crumb homogeneity, and raises the

gluten index in dough A strengthening effect on the gluten network, in particular, causes an increase in dough stability, oven spring, and specific volume, as well as a change in bread crust fracture behaviour. Other applications of this enzyme were discovered in brewer's spent grain, a by-product of the brewing industry with a high fibre content that could be treated with a lipase mixture to increase the expansion of the gluten network, increase wall thickness, and reduce cell density, thereby improving the volume and crumb structure of high-fiber white bread.

Buckwheat flour, a highly nutritious pseudo-cereal high in dietary fibre, was combined with phospholipase; lipid hydrolysis improved dough stability and quality.At the end of the baking process, the higher temperature and lower moisture content induce a non-enzymatic browning reaction, which results in crust formation on the bread's surface. In many bakery products, acrylamide (a chemical compound with possibly carcinogenic effects in humans) is formed due to the presence of reducing sugars and L-asparagine, in addition to high temperatures. Asparaginase reduced acrylamide formation in the crust and crumb regions by 97% and 73%, respectively, without changing the rheological properties or physico-sensory properties of bread, according to Mohan *et al.* (2013). Kukurova *et al.* (2009) observed similar results, reducing acrylamide formation by up to 90%. This enzyme also converted glutamine to glutamic acid, but neither action had any effect on the browning or Maillard reactions**.**

Enzymes Combinations for Enhancing Bread Quality

Numerous enzyme combinations have been used to improve the industrial or instrumental quality of bread. However, before selecting the best or optimal combination of enzymes, it is necessary to understand the potential synergies or antagonisms within them to improve dough performance and baked product quality. Furthermore, a suitable application of enzyme combination provides a wide range of options for improving bread quality. Combinations of a wide range of enzymes are used to improve dough performance and final product characteristics in breadmaking. Some enzyme combinations are either antagonistic or have no effect for the intended purpose. Collar *et al.* (2000) state that combining xylanase with GOX is not recommended because GOX inhibits the softening effect of xylanases. Furthermore, Bilgicli *et al.* discovered limited effects on dough and bread properties when combining two cross-linking enzymes, TGase and GOX, in conjunction with several emulsifiers and ascorbic acid as an oxidant in a wheat-lupin flour blend. In addition to increasing bread volume, it has been reported that GOX works synergistically with phospholipase to ensure roll shape uniformity and tolerance to process changes. Similarly, Selinheimo *et al.* (2006) found that when GOX was combined with laccase, dough hardening was reduced because the latter mediated the depolymerization of the cross-linked arabinoxylans network.

In contrast, ideal enzyme blends with enhanced enzymatic activities have been proposed. Gil *et al.* (1999) found that combining pentosanase, bacterial -amylase, and lipase increased bread crumb elasticity while decreasing firmness during storage for up to 72 hours. An optimal combination of 0.010 percent (w/w) xylanase, 0.005% (w/w) papain, and 0.002% (w/w) xylanase GOX was defined by comparing browning index enhancement in 24 hours using an orthogonal experimental design, and its efficiency was confirmed when used as a rheological improver for fresh whole wheat dough. Immobilized GOX on chitosan (CS)-sodium tripolyphosphate (TPP)

combined with fungal-amylase added to wheat flour resulted in a slight increase in bread springiness and specific volume and a decrease in hardness, improving the final quality of the bread.

The combination of enzymes resulted in a significant improvement in bread quality indicators such as volume, porosity, and elasticity, clearly superior to their individual use. In fact, flour with low alpha amylase activity must be supplemented with additional alphaamylase, but alphaamylase added to weak flour can reduce dough quality. Shafisoltani *et al.* proposed combining GOX and xylanase to improve the quality of wheat flour after it had been optimised with -amylase. The results showed that using this enzyme combination resulted in dough with low stickiness and bread with higher specific volume, better shape, lower crumb hardness, and a higher total score in a sensory evaluation test, but that these effects were dose dependent.

Non-starch carbohydrases are frequently used in tandem. Stoica *et al.* investigated the effect of three hemicellulolytic enzymes in short gluten flours—a fungal hemicellulase from Aspergillus oryzae, a fungal pentosanase from Humicola insolens, and a bacterial xylanase from *Bacillus subtilis*—on physical indices such as bread volume, porosity, and elasticity. The use of an enzymatic cocktail containing xylanase, xylosidase, and alpha Larabinofuranosidase, produced by the thermophilic fungus Thermoascus aurantiacus, on wheat bread confirmed that these enzymes primarily attack water-insoluble arabinoxylan, resulting in an increase in specific volume and a decrease in crumb firmness and amylopectin retrogradation during bread.Hsing *et al.* obtained similar results and recommended a combination of hemicellulase and/or endoxylanase with ascorbic acid, which delayed staling and improved the quality of freshly baked bread.

Gluten-crosslinking enzymes can significantly improve dough's functional properties. Caballero *et al.* investigated the effect of a number of gluten-crosslinking enzymes (transglutaminase, glucose oxidase, and laccase), as well as polysaccharide and gluten degrading enzymes (alpha-amylase, xylanase, and protease) in breadmaking systems. The findings revealed significant interactions within enzymes as well as an improvement in their anti-staling properties. Furthermore, the effect of the pairs glucose oxidase-laccase, glucose oxidase-pentosanase, amylase-laccase, amylaseprotease, and pentosanase-protease on bread quality was significant. Recently, it was proposed that a combination of three lipases added to wheat flour could change the lipid composition of the flour, specifically decreasing the level of galactolipids and phospholipids while increasing the level of 'lyso'-lipids and free fatty acids.

Enzymes as Healthy Aids in Baking Goods

Numerous enzyme combinations have been used to improve the industrial or instrumental quality of bread. However, before selecting the right or optimal combination of enzymes, it is necessary to understand the potential synergies or antagonisms within them to improve dough performance and baked product quality. Furthermore, a suitable application of enzyme combination provides a wide range of options for improving bread quality. Combinations of a wide range of enzymes are used to improve dough performance and final product characteristics in breadmaking. Some enzyme combinations are either antagonistic or have no effect for the intended purpose. Collar *et*

al. (2000) state that combining xylanase with GOX is not recommended because GOX inhibits the softening effect of xylanases. Furthermore, Bilgicli *et al.* (2014) discovered limited effects on dough and bread properties when combining two cross-linking enzymes, TGase and GOX, in conjunction with several emulsifiers and ascorbic acid as an oxidant in a wheat-lupin flour blend. In addition to increasing bread volume, it has been reported that GOX works synergistically with phospholipase to ensure roll shape uniformity and tolerance to process changes.

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frozen storage for overcoming the detrimental effect of bran on mineral bioavailability. The results confirmed that the addition of phytase and the freezing process increased phosphorus bioavailability.

Rice bran was enzymatically treated with an enzyme combination of alcalase, cellulose, and phytase before being mixed with wheat flour to increase potential fibre content. The results showed that either raw or enzyme-treated rice bran was suitable for breadmaking, with improved sensory and nutritional values.

Recent research indicates that microbial TGase could be used to detoxify gluten by selectively modifying glutamine residues of intact gluten via transamidation with lysine methyl ester or by crosslinking gluten peptides in beverages via isopeptide bonds, allowing them to be removed via filtration. Other enzymes, such as commercial preparations of laccase, glucose oxidase, and protease, have been tested in gluten-free breads (Renzetti et al., 2010). Protease has also been studied in rice flour and different commercial gluten-free flours (buckwheat, corn, sorghum, and teff) individually (KawamuraKonishi et al., 2013) or in combination with glucose oxidase (Renzetti and Arendt, 2009) to evaluate their impact on breadmaking performance. In this trend, the addition of various groups of enzymes in gluten-free bread production was investigated in order to improve final product quality, primarily by crosslinking proteins (Rosell, 2009). Basso *et al.* recently investigated the potential use of cyclodextrin-glycosyltransferase (CGTase) enzyme produced by Bacillus firmus strain 37 in breadmaking and the development of gluten-free bread with pinion and corn flours. The addition of CGTase improved the bread's specific volume, texture, and sensory characteristics cyclization is the specific enzymatic reaction that releases cyclodextrins (CDs), the most common being CDs and CD which consist of six, seven, and eight glucose monomers in cycles, respectively.

Corn starch samples were modified with CGTase below the gelatinization temperature to produce porous, partially hydrolyzed granules with CDs that could be used in place of modified corn starches in food applications. The benefits of containing primarily -CD influence pasting behaviour and may impede amylase orientation, slowing hydrolysis, which may help mice maintain lower blood glucose levels. The presence of CDs in a food formulation to improve or modify the characteristics of the final product, which may result in starch samples that are more slowly digested, is granted GRAS (Generally Recognized as Safe) on wheat bread confirmed that these enzymes primarily attack water-insoluble arabinoxylan, resulting in an increase in specific volume, a decrease in crumb firmness, and amylopectin retrogradation during bread storage. Hsing *et al.* obtained similar results and recommended a combination of hemicellulase and/or endoxylanase with ascorbic acid, which delayed staling and improved the quality of freshly baked bread.

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CGTase has also been used to treat natural and extruded wheat flours (Román et al., 2016). The level of released CDs revealed that -CD was the most abundant, followed by -CD, with very low -CD values obtained most likely due to formation.CD-lipid complexes are a type of CD-lipid complex. CDs are non-toxic ingredients that are completely metabolised by the colon microflora after they are not absorbed in the upper gastrointestinal tract.They have found widespread application in the food and pharmaceutical industry. The presence of CDs in cereal-based products may open up new avenues for obtaining a healthy product with an added benefit.

CHAPTER 8

ENZYMES IN WINEMAKING

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The biological winemaking process is the result of a series of biochemical transformations caused by the action of several enzymes. Many of these enzymes are derived from the grape, the indigenous microflora on the grape, and the microorganisms present during the winemaking process. Furthermore, because the endogenous enzymes of grapes, yeasts, and other microorganisms present in must and wine are frequently inefficient or insufficient under winemaking conditions, commercial enzyme preparations are widely used as supplements.This chapter focuses on the most common commercial preparations of enzymes used in oenology, some of which are widely used by winemakers (e.g., pectinases), while others are not (urease).

Enzymes to Improve Maceration

Color is one of the most important aspects to consider when evaluating red wine. High perceived quality scores are typically associated with dark-colored wines. Because these molecules and their interactions with other phenolic compounds are responsible for formation and its stability during ageing, anthocyanins contribute significantly to the sensory quality of red wines. Anthocyanins are primarily found within skin cell vacuoles and are partially extracted from the berry skin into the must/wine during the winemaking process.

The anthocyanin content and composition of red wines are determined by the amount of pigments in the berry skin at harvest as well as the ease with which they can be extracted. Although the qualitative and quantitative composition of anthocyanins in wine is directly related to wine grape variety, ripening stage, culture practises, growing season, and environmental conditions, oenological practises are also important (Figure 8.1)

Exogenous enzymes are commonly used in red winemaking to accelerate the extraction of anthocyanins from the berry skin and thus increase the colour intensity of the resulting wine.Commercial enzyme preparations primarily exhibit pectolytic (polygalacturonase, pectin methyl esterase, and pectin lyase) cellulase, hemicellulase, and acid protease activities by partially hydrolyzing structural polysaccharides, maceration enzymes degrade the berry skin pecto-cellulosic cell walls. As a result, the cell wall's permeability increases, facilitating the diffusion of anthocyanins from vacuoles into the must during fermentation. The impact of maceration enzymes on anthocyanin content and colour intensity in red wines has yielded contradictory results. The differences are most likely due to the differences in the nature and activities of the commercial enzyme preparations, as well as varietal and vintage effects on grape anthocyanin content and composition or skin cell wall morphology and composition..

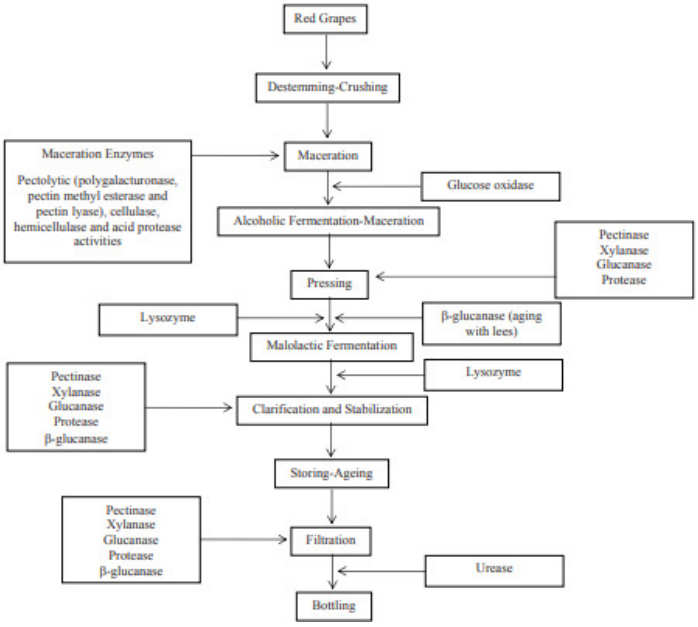

Figure 8.1 Represent the Scheme red wines. Typical production process of red wines showing the stages at which enzymes are added.

Differences in mechanical properties of berry skin are also linked to differences in the chemical composition of cell walls, which determine the skin's resistance to anthocyanin release. Berry skin hardness and thickness, in fact, influence the rate and extent of anthocyanin extractability. Although it is well known that cell wall degradation causes skin softening, the effect of maceration enzymes on the mechanical properties of the berry skin has only been quantified to date by Ro-Segade *et al.* who discovered that enzyme preparation influenced the mechanical properties of the berry skin, increasing the softening that naturally occurs during maceration as a result of the degradation process. The effect of enzymes on skin hardness was measured experimentally for the first time, and mechanical properties of the skin may be used to predict anthocyanin extraction yield during maceration. Furthermore, the use of enzymes allowed for an increase in anthocyanin extraction yield, a reduction in maceration time, and the prevention of anthocyanin loss during maceration. However, depending on the anthocyanin composition of wine grapes, a variety effect was discovered.

The release of anthocyanins, chromatic characteristics, and colour stability in red wines are all affected by maceration. A longer maceration time usually results in more anthocyanin extraction from the skins and improves the wine's colour stability. However, the participation of extracted anthocyanins in oxidation and polymerization reactions occurring during the maceration process, their partial adsorption by yeasts, and their fixation on grape solid parts may all have an impact on this relationship.Another factor to consider in red winemaking is the maceration temperature. Traditional red winemaking is distinguished by the development of alcoholic fermentation and maceration at the same time. New winemaking techniques have been proposed that take into account the selective effect of maceration conditions on grape component extraction and the reactions in which they participate (Martn and Morata de Ambrosini, 2014). Pre-fermentation

Cold Maceration (PCM) is a method used prior to fermentation to encourage extraction in an aqueous medium at a low temperature (5-15°C), resulting in preferential solubility of water-soluble compounds and encouraging selective extraction of anthocyanins and tannins with low molecular weight. Furthermore, it produces a better-structured product that is higher in phenolic and aromatic compounds, thereby maintaining a strong relationship with the production area.

Fermentations at low temperatures (15-20°C) on the other hand, can increase the production and retention of volatile compounds, thus improving the aromatic profile of wines (Molina et al., 2007). White winemaking has traditionally been done at low temperatures, and red winemaking is catching on. Low maceration temperatures, on the other hand, can reduce colour extraction in red wines—an effect that can be mitigated by using cold-active pectinolytic enzymes, which ensure good colour extraction at low temperatures.

Martn and Morata de Ambrosini (2013) described a cold-active pectinolytic system derived from a psychrotolerant Bacillus sp. that increased colour extraction in low-temperature short macerations with red grape skins.Recently, the same authors (Martn and Morata de Ambrosini, 2014) demonstrated that a pectinolytic enzymatic system derived from the Bacillus sp. CH15 bacterial strain greatly accelerated colour extraction by shortening the PCM stage and reducing the maceration time required for winemaking at low temperatures. At devatting and after six months of storage, enzyme-treated wines had better chromatic metrics than controls. The cold-active enzyme compensated for the reduction in colour extraction caused by the low maceration temperature, resulting in high-quality wines with chromatic characteristics similar to traditional wines.

Enzymes to Improve Clarification, Filtration and Yield of Juice and Wine

Since the 1970s, winemakers have used commercial enzymes (pectinases, xylanases, glucanases, and proteases) to improve clarification and filtration and to increase pressing efficiency and juice extraction.However, the number and variety of products available, as well as our understanding of their action mechanisms and impact on wine quality, have increased dramatically in recent years.The most important enzymes in winemaking are pectinases. Pectic compounds, along with cellulose, hemicellulose, and lignin, form part of the grape cell wall and act as adherents between cells, giving the cell wall consistency (Bisson and Butzke, 1996; Vidal et al., 2003). The rupture of these cell structures promotes the extraction of substances found in the solid part of the grape, primarily the pulp and skin. Pectolytic enzymes degrade these compounds, enhancing the extraction and clarification of the must. They may also facilitate the extraction of substances that affect the aroma and colour of the skin and pulp.All of these commercial enzyme preparations are derived from microorganisms grown on substrates under conditions that optimise production and facilitate purification at a reasonable cost.

The majority of commercial preparations are derived from various species of filamentous fungi, primarily Aspergillus spp., which are GRAS (Generally Recognized As Safe) and are included in the International Code for Enological Practices of the Office International of Vine and Wine (O.I.V.). Many of them are enzyme blends that perform multiple functions in the process. Preparations based on pectinase, cellulase, and hemicellulase enzymes are the most commonly

used in winemaking to improve juice and wine clarification, filtration, and yield. Commercial preparations frequently have unwanted concomitant or side effects in addition to desirable effects.

These effects may include the oxidation of phenolic compounds during maceration (Fernández-Zurbano et al., 1999) and the production of methanol due to the presence of pectinmethyl-esterase activity in various pectolytic preparations (Revilla and González-San José, 1998), or even the enhancement of volatile phenol production due to the presence of cinnamyl-esterase activity in most enzymatic preparations. Other side effects include significant protein and glucidiccolloid loss. As a result, it would be advantageous to produce these enzymes at a low cost in order to develop novel applications. The current trend is to look for alternative sources of pectinases, particularly from yeasts. In the production of pectinase on a large scale, yeasts have an advantage over filamentous fungi. Some research has already been conducted to determine whether yeast proteolytic enzymes can be used in the food industry. GRAS yeasts Saccharomyces and Kluyveromyces are the most studied and appear to be the most promising. As a result, their pectinases can be used to clarify fruit juices and wines while keeping methanol levels low.

Gainvors *et al.* demonstrated in 1994 that a crude pectolytic extract from a *S. cerevisiae* strain had the same effect on turbidity as the same amount of a commercial pectinase preparation when added to grape must. Blanco *et al.* demonstrated that when wine fermentations use pectolytic strains of *S. cerevisiae*, the clarification process is highly facilitated, with filtration time reduced by up to 50% in some cases.According to Pretorius pectolytic wine yeasts may improve the liquefaction, clarification, and filterability of grape must by releasing more colour and flavour compounds entrapped in the grape skins and thus contributing to the wine bouquet. Fernández-González *et al.* transformed a good enological wine yeast strain with the yeast PGU1 gene fused to the promoter of the PGK1 gene in order to increase polygalacturonase (PG) expression during vinification. Microvinification assays on white and red musts treated with this transmogrified strain revealed a significant increase in must/wine extraction yield. However, Radoi *et al.* (2005) discovered that pectolytic recombinant strains were no more efficient in winemaking than pectolytic wild-type strains.

1 β-glucanases to improve clarification and filtration

Glucans produced by Botrytis cinerea in botrytized grape juice are the polysaccharides with the greatest influence on the clarification and stabilisation of must and wine. -glucan fragmentation is aided by Hydrolytic enzymes (e.g., -glucanases) can improve water solubility by reducing polymerization. β-D-glucans are constituted by a main chain formed by 3-D-glucose units joined together by β-1,3 glucosidic bonds in yeasts and fungi, such as *Saccharomyces cerevisiae*, S. bayanus and Botrytis cinerea or β-1,4 glucosidic bonds in cereals and by a variable number of lateral chains with different lengths that are linked to the main chain by β-1,6 or β-1,2 bonds. -Glucanases are a diverse class of enzymes that include both exo/endo forms of -(1,6)-glucanases capable of promoting the hydrolysis of -(1,6)-O-glycosidic bonds connecting lateral branches with the main polymeric chain of -glucans, as well as exo-(1,3) and endo-(1,3)-glucanases capable of promoting the lysis of terminal and internal (Venturi et al., 2013). Commercial

preparations of these enzymes are widely used in the brewing and winemaking industries to aid in the filtration of musts and wines, particularly those derived from B. cinerea-affected grapes. At the end of the alcoholic fermentation, -glucanase preparations are also used to promote the lysis of the yeast cell walls, followed by the release of their main constituents, including manno-proteins and oligomeric fragments of beta glucans.

Assisted Wine Aging on lees with β-glucanase

Traditionally, only white and sparkling wines were made in contact with their lees. Nonetheless, this technology has recently been extended to other types of wine, such as red wines, because it is thought to improve consumers' organoleptic perception of wines. After the alcoholic fermentation is complete, these wines are aged on lees for a variable period of time depending on their characteristics. Lees are primarily composed of microorganisms (yeasts and bacteria), with tartaric acid and inorganic matter playing a minor role (Perez-Serradilla and de Castro, 2008). The autolysis of wine yeasts occurs during lees ageing, changing the chemical and sensory characteristics of the wine. Yeast autolysis is the hydrolysis of biopolymers by hydrolytic enzymes (such as -glucanases and proteases), which releases various compounds into the wine (Pozo-Bayon et al., 2009). Peptides, amino acids, fatty acids, and nucleotides are derived from yeast cytoplasm, whereas glucans and manno-proteins are derived from yeast cell wall. These compounds have a significant impact on the organoleptic properties and stability of wines, as well as the foaming of sparkling wines.

The period of ageing on lees varies according to the desired wine characteristics, though a longer time contributes to the wine's quality (Rodriguez-Nogales et al., 2012a). Enzymatic preparations rich in b-glucanases can be used to reduce this long period, and thus the risks of oxidation and microbial contamination, as well as the high production costs. Exogenous glucanases can catalyse the hydrolysis of the cell wall b-glucan chains' b-(1-3) and b-(1-6)-glycosidic bonds, gradually degrading the cell wall and accelerating yeast lysis.According to some studies, this enzymatic treatment in wines ageing on lees did not cause any significant changes in their basic chemical composition or phenolic compounds, neutral and total polysaccharides, proteins, and colour in red wines.

There is little information available on the effect of adding exogenous b-glucanases to sparkling wine. There was a slight increase in the level of free amino, but this treatment had no significant effect on the total protein content or the foam characteristics (Torresi et al., 2014). Rodriguez-Nogales *et al.* discovered that enzyme-treated wines had a higher content of neutral polysaccharides, as well as more volume, flavour intensity, yeast and dough smell, and a sweeter taste. Furthermore, it has been demonstrated that adding b-glucanases to sparkling wines increases their antioxidant properties.

Enzymes for Enhancing Wine Aroma

Grape varietal aroma consists of volatile free odour substances (primarily monoterpenes, C13-norisoprenoids, benzene derivatives, and long-chain aliphatic alcohols) and odourless non-volatile glycoconjugates. Glycosidically bound volatiles include glucose as well as rhamnose, arabinose, and apiose in disaccharides glycosides. Using exogenous glycosidases, these

glycosidic aroma precursors can be converted into odorous volatiles. This procedure typically involves a two-step mode reaction.

First, depending on the sugar moieties of the substrates, a-rhamnosidase, a-arabinosidase, or b-apiosidase releases the terminal sugar; second, a b-glucosidase releases the aromatic aglycone and glucose.The glycosidic aroma precursors have been shown to be stable during winemaking operations because the effect of grape and *S. cerevisiae* glycosidases is very limited (Maicas and Mateo, 2005). The selection of active yeast, fungal, and bacterial glycosidases to enhance wine aroma has been extensively researched for this purpose. Aspergillus niger commercial preparations rich in glycosidases are currently available. These fungal enzymes are active in winemaking conditions, but due to glucose inhibition, they must be added at the end of fermentation.

Commercial preparations must be free of cinnamate esterase activity in order to avoid the synthesis of off-aromas (such as vinylphenols) in a synergistic reaction with yeast decarboxylation. Furthermore, the use of these commercial preparations must be restricted to white wines in order to avoid colour loss due to the hydrolysis of anthocyanins to anthocyanidins. Wang *et al.* discovered a b-glucosidase from Trichosporon asahii that has little effect on the major anthocyanins, allowing it to be used in red wines.

Even with a wide range of enzymatic activities, the effectiveness of the treatment using commercial glycosidases is highly dependent on the aromatic potential of the grape. It has been proposed that prior glycoside enrichment of wine via skin-contact treatment of the must, followed by the addition of glycosidic precursors, significantly improved the wine's volatile composition.

Volatile thiols are also potent odoriferous compounds found in wine made from various grape varieties. The volatile thiols with the most odorant impact in wines are mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan1-ol (3MH), and its acetate (3MHA). Commercial yeast strains of *S. cerevisiae* have been shown to release these volatile thiols via b-lyase activity from the odourless precursors (cysteinylated and glutathionylated precursors) of the juices. However, their effectiveness is severely limited. The use of exogenous b-lyase could theoretically improve the wine's volatile thiols; however, no commercial enzymatic preparations are currently available.

Protein Haze Prevention Using Proteolytic Enzymes

Proteins, along with polysaccharides and polyphenols, are important components of grape juice and white wine. Some of these proteins are responsible for white wine sediments and haze when affected by inadequate storage. The haze-forming proteins have been identified as pathogenesis-related (PR) proteins, with chitinases, thaumatin-like proteins (TLPs), and b-glucanases being the most common. It has also been suggested that the pH of the wine plays a significant role in this issue.

The mechanism of protein haze formation has recently been updated (Van Sluyter et al., 2015), and it consists of three stages. The increase in temperature causes the proteins to unfold and

expose their hydrophobic binding sites in the first stage. The unfolding proteins self-assemble in the second stage via hydrophobic interactions. Finally, in the third stage, the protein aggregates gradually become crosslinked as a result of the actions of sulphates and polyphenols, flocculating into a hazy suspension and eventually formatting precipitates.The most common strategy used by winemakers to prevent wine haze is bentonite treatment. Nonetheless, the use of bentonite has been linked to a negative impact on wine aroma and flavour, colour loss, wine volume loss, high handling costs, and the environmental impact of the residues.Different strategies to prevent protein haze have been studied in order to reduce or eliminate the drawbacks of bentonite. However, the success of these treatments has not been fully realised, whether in terms of their ability to eliminate haze or their negative effects on wine quality.

The use of proteolytic enzymes to hydrolyze haze-forming proteins is an appealing alternative. Proteases catalyse the cleavage of hydrolytic bonds within proteins, allowing peptides and/or amino acids to be released. It should be noted that proteases for oenological applications must function under extreme conditions such as acid pH of 3-4, the presence of inhibitors, and low temperatures.

Furthermore, the haze-forming proteins have a high resistance to enzymatic hydrolysis due to their structure. Chitinases and TLPs are both small, compact enzymes with globular structures. Under winemaking conditions, the attack of the proteases is complicated due to these specific circumstances.One of the most important issues is selecting an appropriate source of proteases. Grapes, yeast, bacteria, and spoilage microbes are all potential sources of active proteases in the winemaking environment. Grape endogenous proteases are active at wine pH, but they have a low degree of proteolysis.

The main wine yeast, S. cerevisae, does not normally secrete external proteases, but several studies show that some strains do. During grape juice fermentation, *S. cerevisiae* PlR1 demonstrated exo-proteolytic activity, though it appears to be insufficient to counteract the effect of PR proteins. However, there are numerous examples of acid-tolerant proteases secreted by non-Saccharomyces yeasts, but these enzymes have not been thoroughly characterised under winemaking conditions.

Glucose oxidase to reduce Wine Alcohol content

Wine is an alcoholic beverage made from fermented grape juice that contains a high amount of water and alcohol. The alcohol concentration influences the wine's ageing, stability, and organoleptic properties. However, high levels of alcohol can mask the sensorial perception of some volatile compounds, induce the perception of hotness, body, and viscosity, and promote the perception of bitterness. High alcoholic wines may be perceived as unhealthy products by consumers.

Climate change is causing unbalanced grape ripening in later years.

As a result, wines with high ethanol concentrations are produced (VilaCrespo et al., 2010). Thus, winemakers are keenly interested in developing strategies to reduce excessive ethanol content in wine in order to improve wine taste balance. On the other hand, there is a growing market for the

production of dealcoholized (> 0.5% v/v), low-alcohol (0.5-1.2%), and reduced-alcohol (1.2-6.5%) wines (Pickering, 2000).

Many techniques have been tried to reduce or eliminate the alcohol content of wine. Some of them necessitate the reduction of fermentable sugars via early grape harvest, juice dilution, membrane filtration techniques, or the use of glucose oxidase enzyme (GOX). Others concentrate on the fermentation process, employing modified yeast strains with lower ethanol production.Finally, there is a third group of post-fermentation techniques that use membrane transport processes (reverse osmosis, pervaporation, and osmotic distillation) or non-membrane extractions (supercritical solvent extraction and spinning cone column) to remove wine alcohol.As previously stated, the use of GOX is an alternative to depleting the sugar level in grape must, resulting in wines with lower alcohol after fermentation with native or selected yeasts, typically *S. cerevisiae*.

GOX is a flavoprotein that uses molecular oxygen as an electron acceptor to catalyse the oxidation of b-D-glucose to D-gluconod-lactone and H_2O_2. D-glucono-dlactone is hydrolyzed non-enzymatically to gluconic acid, and then the flavine adenine dinucleotide (FAD) ring of GOX is reduced to FADH2 (reductive half reaction).Oxygen reoxidizes the reduced GOD to produce H2 O2 (oxidative half reaction). In most commercial GOX preparations, a second enzymatic activity (catalase) is present to break down the reaction byproduct.

The use of GOX in various grape varieties has been investigated. The fermentable sugar fraction in grape must is approximately 50% glucose and 50% fructose. The total depletion of glucose using GOX could theoretically result in wines with approximately half the potential alcohol content.However, the reduction in alcohol levels reported in GOX-treated musts ranged from less than 4% to 40%, because the efficiency of the enzymatic process is dependent on enzyme concentration, must pH, dissolved oxygen concentration, reaction time, and temperature. Due to the presence of large amounts of gluconic acid, the GOX-treated wines had higher acidity than the control wines in terms of chemical and sensorial characteristics. These wines also had a higher colour intensity, fewer fruit aromas, and a longer flavour length. Furthermore, higher concentrations of esters and fatty acids were reported, with little change in the concentrations of the other volatile compounds. A refined method for the production of wines with lower content of alcohol, involving the treatment of the unfermented grape juice with GOX and glucose isomerase, has been recently reported. Despite the benefits of using GOX to reduce the alcoholic degree of wine mentioned above, the International Organization of Vine and Wine does not currently recognise this technology.

Glysozyme in Winemaking

Lysozyme is a muramidase enzyme derived from hen egg white that can be used to regulate malolactic yeast (MLF) during winemaking. This enzyme hydrolyzes the cell wall of Gram-positive bacteria that are susceptible.bacteria, increasing permeability and causing the cell to burst (Chassy and others)

Denaturation, attachment of other compounds, and the use of membrane-permeabilizing agents have all been tried to broaden the antimicrobial spectrum of lysozyme to Gram-negative bacteria

(Masschalck and Michiels, 2003). In the European Union (EU) and several other countries, hen egg white lysozyme (HEWL) is considered a natural antimicrobial and is approved as a food preservative.MLF is a bioconversion of malic acid into lactic acid by lactic acid bacteria that occurs in red and some white wines. However, some of the MLF species can also cause biogenic amine formation, wine spoilage, and sluggish or stuck fermentations. Several authors have investigated the role of LAB in wine and the use of lysozyme against LAB in winemaking.

In this regard, lysozyme is being used as an alternative to sulphur dioxide to control LAB proliferation in wine; thus, regulation EC No. 2066/2001 allows up to 500 mg/L of HEWL to be added to wine or must. Lysozyme can be used in winemaking for a variety of purposes, including inhibiting MLF development, reducing competition between yeasts and bacteria, reducing the occurrence of sluggish and stuck fermentations, and microbiological stabilisation of wine after MLF.After HEWL wine treatment, negative effects on wine composition parameters have been reported. Nonetheless, the same authors discovered that different wine components were responsible for the inhibition of several enzymes in wine, including lysozyme.

Lysozyme contains a well-known major allergen derived from hen's eggs. Several studies have shown that HEWL can be considered a possible allergen, and because of this allergenicity, lysozyme must be labelled on wine bottles if it is present at a trace level in the final product. Taking this into consideration, Liburdi *et al.* focused on lysozyme immobilisation and developed an integrated perspective on how to use and customise the enzymes for their specific wine application.

CHAPTER 9

UREASE IN WINE

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Urea in wine and other alcoholic beverages is a precursor of ethyl carbamate (urethane, EC), which has shown potential carcinogenic activity in animal tests when administered in high doses (Schlatter and Lutz, 1990). The presence of urea is related to the metabolic activity of the various microorganisms present in the winemaking process, including yeasts and bacteria.

Among the various preventive actions to reduce EC levels, acid urease (urea amidohydrolase) hydrolysis appears to be a suitable process to prevent EC formation. Ureases are a class of highly efficient enzymes found throughout nature that catalyse the hydrolysis of urea, producing carbonic acid and ammonia as byproducts (Krajewska, 2009). Acid ureases are a distinct subgroup of ureases that, unlike typical (neutral) ureases, have optimal pHs in the range of 2-4.5, allowing them to be used in wine. Lactobacillus fermentum produces oenological urease. If a wine contains more than 3 mg/l of urea, it must be carefully incorporated and mixed in order to be aged for more than one year, according to the International Oenological Codex. After a significant decrease in urea, for example, less than 1 mg/l, all enzymatic activity is removed by filtering the wine (pore diameter less than 1 m).Several authors have investigated various technical details in order to improve the oenological use of urease through enzyme optimization. Others have concentrated on the kinetics of urea degradation in various stirred bioreactors.

Cassava Fermentation

Cassava (Manihot esculenta Crantz) is a major root crop in a number of tropical and subtropical countries, including Latin America, Asia, and particularly West Africa. Cassava, also known as manioc, mandioca, tapioca, or yucca, is a cyanogenic food crop. Cassava is also regarded as a hardy crop that thrives in harsh conditions where few other crops can thrive (Cach et al., 2006). Cassava is drought tolerant and resistant to most diseases and pests.Furthermore, cassava is a staple food for over 500 million people in over 90 countries, and it is the third most important source of calories in the tropics, after rice and corn.

However, cassava toxicity occurs due to the presence of cyanogenic glucosides or cyanogens (naturally occurring substrates), specifically linamarin (2-hydroxy-isobutyronitrile/-D-glucopyranoside) and lotaustralin (2-hydroxyl-2metylbutyro-nitrile/-D-glucopyranoside) (Fig. 1), both of which have fatal consequences when consumed in unprocessed food. Furthermore, these substrates (cyanogens) can cause cyanide poisoning, which causes dizziness, stomach pain, headache, nausea, vomiting, and, in rare cases, death. Furthermore, daily consumption of cassava food products containing residual levels of these cyanogenic glucosides (cyanogens) can cause cretinism, goitre, tropical ataxic neuropathy, and tropical diabetes (Ahaotu et al., 2013;

Ogbonnaya, 2016). The World Health Organization has recommended that the maximum safe intake of cyanide-containing food/feed for humans and animals be 10 mg HCN/kg body weight. As a result, significant efforts are made to reduce/remove the cyanogenic glucosides through the use of endogenous or exogenous (microbial) linamarase (-glucosidase) enzymes that release the toxic cyanohydric acid. Traditional methods, such as spontaneous fermentation, are used to remove cyanohydric acids from cassava tubers so that they can be consumed by humans (Murugan et al., 2014). These enzymes convert cyanide-containing compounds into acetone cyanohydrins, which decompose spontaneously to hydrogen cyanide (HCN) (Fig. 2). HCN is either released into the atmosphere or readily dissolves in water.

Microbial Linamarase (β-glucosidase)

Linamarase (EC 3.2.1.21; -D-glucoside glucohydrolase) is a well-studied -glucosidase (hydrolase/cellobiase) that catalyses the hydrolysis of glycosidic linkages via glycosyl group transfer. Linamarase (native hydrolase) is derived from crop-like cassava and microbial sources (exogenous linamrase). Endogenous linamarase is primarily responsible for linamarin and lotaustralin detoxification in cassava.Yeast and lactic acid bacteria (LAB) are microbial strains that have been linked to the production of linamarase during cassava fermentation and flavour development. Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus brevis, Leuconostoc pseudomesenteroides, Lactobacillus fallax, and Weissella paramesenteroides are the most common LAB-producing linamarase producers for the preparation of traditional cassava food. Other major LAB included Streptococcus spp. and Corynebacterium spp., the numbers of which decreased with fermentation time. However, linamarase enzyme was found in a number of yeasts isolated from fermenting cassava, and this has been linked to detoxification of cyanogenic glucosides during cassava fermentation.

Nwokoro and Anya (2011) investigated cassava cyanide degradation as well as the biochemical properties and purifying of linamrase enzyme from Lactobacillus delbrueckii NRRL B-763. After 20 hours of treatment, the crude enzyme decreased from 2.1 mg HCN/10 g sample to 0.11 mg/HCN/10 g sample (a 95% reduction). Untreated control samples, on the other hand, showed a reduction of only 5.7% from 2.1 mg HCN/10 g sample to 1.98 mg HCN/10 g sample after 40 hours of incubation. The enzyme was purified 33 times with a 40% yield and demonstrated maximum activity at pH 4.5.Murugan *et al.* isolated cyanogenic glucoside from cassava peel using indigenous bacteria, *Bacillus subtilis* KM05, and evaluated its potential for cyanogen detoxification. The enzyme linamarase (53 KDa) was partially purified from the *B. subtilis* KM05 strain and its activity was determined to be 9.6 U/ml. It was also deduced that the enzyme could effectively degrade cynogens.

Ogbonnaya (2016) investigated the hydrolysis of cyanides in cassava flour by cassava endogenous and microbial enzymes. Eight linamarase-producing bacteria were isolated (Lb. plantarum, Lb. fermentum, Pounds. amylovorus, Lb. cellobiosus, Lc.mesenteroides, Pseudomonas stutzeri, Bacillus pumilus, and *B. subtilis*).Cassava tubers and soil samples were identified and isolated. All of the isolates were grown in media containing Tween 80 solution, a surfactant. The Lb. fermentum produced the highest enzyme activity (6.82 U/mL) in the surfactant-containing medium. After 30 hours, the cassava flour containing residual cyanides

was found to be undetectable when treated with linamarase from Lb. fermentum. However, after 80 hours, the residual cyanides in cassava flour treated with endogenous cassava linamarase had been found to be 0.39 mg/10 g cassava flour. The results showed that fermenting cassava tubers with isolates provided better control over the cassava fermentation, potentially leading to the production of non-toxic cassava food products.

Linamarase in Fermentation Processes

Fermentation-aided linamarase is critical for improving product quality and safety, particularly by reducing toxic cyanogenic glycosides in cassava food products (Kostinek et al., 2005). Furthermore, fermentation is an essential means of processing in order to improve textural quality, palatability, and nutritive value by enriching with protein. Furthermore, because of its low technology and energy requirements, the cassava fermentation process is widely used to transform and preserve the unique organoleptic qualities of the final product.Cassava processing methods include grating, drying, soaking, boiling, and fermenting whole or fragmented rhizome. However, the fermentation process can be divided into two categories: submerged (involving soaking in water, such as fufu) and solid state.

Submerged fermentation

SmF is a process that involves the growth and anaerobic or partially anaerobic decomposition of carbohydrates by microorganisms in a liquid medium with plenty of free water (Ray et al., 2007). Ezekiel *et al.* investigated the performance of Trichoderma viride (ATCC 36316) in protein-enriched cassava peels by SmF, as well as the effect of enzyme pre-treatment on the enriched product. After three to four days of fermentation, the crude protein content of cassava peels increased eightfold (4.2 to 37.6%).

Cassava fermented food products through SmF

The content of cyanogenic glucosides in cassava tubers and freshly harvested leaves ranges between 137 and 1515 ppm. The cyanogenic glucosides content of cassava tubers and leaves is reduced by 70 to 75 percent after traditional fermentation.Lambri *et al.* (2013) investigated a drying procedure with and without fermentation for cyanogen removal in cassava tubers (pressed pulp). The fermentation process was carried out in the presence of *Saccharomyces cerevisiae*, and detoxification was found to be effective. In drying conditions, a temperature of 60oC, even for a short period of time (say, eight hours), reduced the cyanide content (by 90%).However, the dehydration followed by fermentation process removed the most cyanide content.Several studies have been conducted to identify the microorganisms associated with submerged cassava fermentation, and the various traditional fermented products are listed below.

Fufu

Fufu is a traditional fermented cassava food product that is native to most Nigerians in the south and east (Achi and Akomas, 2006). It was ranked second only to gari (discussed later), which is a fermented cassava tuber mash or dry powder. It is made by steeping whole or cut tubers in water for three to five days, depending on the ambient temperature.

A variety of organisms aerate the mash during fufu production. Fufu is a fermented cassava food that comes in the form of a wet mash or a dry powder (Umeh and Odibo, 2014). It is made by immersing whole or cut tubers in water for three to five days, depending on the temperature.Lafun is a fermented cassava food product (fine powdery) that is widely consumed in West Africa, primarily in Nigeria and Benin. It is made by peeling and cutting cassava tubers into pieces, then soaking them in water (stationary water or stream) for three to four days. The water-soaked peeled tubers are processed by smF, which causes acidification and softening.

Padonou *et al.* used genotypic and phenotypic methods to investigate microorganisms associated with lafun fermentation, such as LAB, aerobic bacteria, and yeast. Microorganisms that occur during lafun production include LAB such as Lb. fermentum, Lb. plantarum, and Lb. fallax, as well as aerobic bacteria such as *B. subtilis*, B. cereus, Pantoea agglomerans, and yeasts such as *S. cerevisiae*, Hanseniaspora guilliermondii, Pichia scutulata, P. rhodanensis, P.

Solid state fermentation

SSF is a process that occurs on the surface of solid surfaces and involves microbial growth and product formation. This process occurs in the absence of 'free' water, and moisture is absorbed into the solid matrix. Many processing methods were developed in recent years by the cassava-farming population.One of these involves the use of microbial linamarase in the SSF method of processing. This process not only reduces the linamarin content, but it also causes effective root softening of cassava flour due to the microflora. Esser and Jurgens investigated the effect of six individual strains (microflora) on cyanogen levels in SSF fermenting cassava. Geotrichum candidum, Mucor racemosus, Neurospora sitophila, Rhizopus oryzae, and Rhizopus stolonifer, as well as a Bacillus sp., were all inoculated into cassava flour for 72 hours. According to the findings, both fermentation duration and microbial growth were involved in lowering cyangonic glucoside levels (62.7%) and changing the cyanogen composition of cassava flour.The enzyme increases cell-wall degrading activity while improving contact between endogenous linamrases and linamrain, which is the most important mechanism of microbial linamarase in the cassava detoxification process.

Cassava fermented food products through SSF

Cassava tubers are highly perishable and start to deteriorate after two days. Furthermore, suitable methods for storing cassava tubers in fresh form are scarce. One approach is to turn the tubers into high-quality cassava flour. As a result, it provides a method for preserving cassava tubers, which are then used for both industrial and traditional purposes.

Cassava tubers are grated, pressed, and dried or toasted to make cassava flour. Akindahunsi (1999) studied the pure strain of Rhizopus oryzae in the fermentation of cassava pulp, which was then processed into flour. The protein content of flour increased to 97 percent. However, the anti-nutrient factors, such as tannin (flour, 0.16 mg/100 g) and total cyanide (flour, 17.21 mg/kg), were significantly low.Oboh and Akindahunsi (2003) discovered that the presence of S. cerevisae increased the nutritional quality of cassava flour, such as protein and fat content. In the SSF condition, the nutritional quality was determined. The results showed that protein (10.9%) and fat (4.5%), but not cyanogens (9.5 mg/kg), content increased in cassava flour (Figure 9.1).

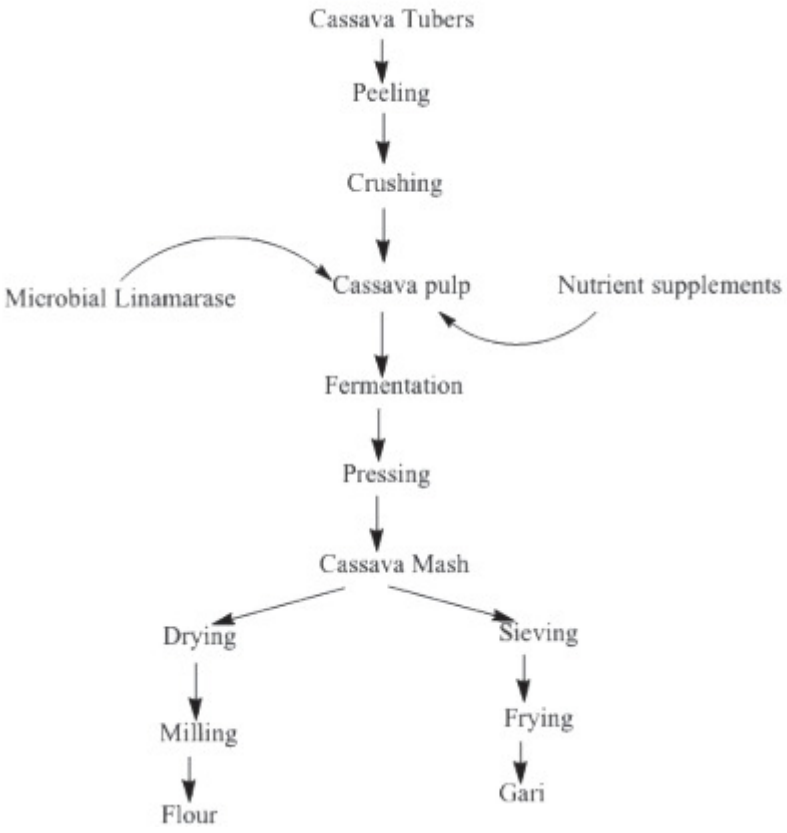

Figure 9.1 Represent the Production chart for solid media.

Cassava fermentation is primarily caused by LAB, Lactobacillus sp., and to a lesser extent Streptococcus sp., which produce acid and gari flavour (Ngaba and Lee, 1979). Yao *et al.* (2009), on the other hand, reported that the strains Lb. plantarum VE36, G2/25, Lb. fermentum G2/10, and Weissella parameseneroides LC11 were chosen to be developed as freeze-dried starter cultures in garri production.Gari is a popular beverage among Nigerians (Asegbeloyin and Onyimonyi, 2007). It is commercially prepared by various microorganism consortia. Akindahunsi (1999) studied the pure strain of Rhizopus oryzae in the fermentation of cassava pulp, which was then processed into gari. The protein content of gari increased by 53% in value.

However, the anti-nutrient factors in gari, such as tannin (0.08 mg/100 g) and total cyanides (14.85 mg/kg), were significantly low.S. cerevisae was used in cassava pulp SSF to improve the nutritional quality of gari. The resulting gari was tested for protein and fat content, as well as anti-nutrient availability.The findings revealed that the protein (6.3%) and fat (3.0%) contents had increased significantly. In contrast, the cyanide level (9.1 mg/kg) decreased significantly.

Oguntoyinbo and Dodd studied the microbial dynamics and diversity of cassava gari production during SSF in West Africa. Several advanced molecular techniques were used to monitor bacterial dynamics during cassava fermentation, including 16S rDNA gene sequence analysis and pulsed field gel electrophoresis (PFGF). Lactic acid bacterial species and their close relatives were identified, including Lb. plantarum, Lb. organisation‘s, Lb. pentosus, Lb. acidophilus, and Lb. casei.

Ahaotu *et al.* investigated the fermentation of un-dewatered cassava pulp by microbial linamarase (Alcaligenes faecalis, Lb. plantarum, Leuconostoc cremoris, and Geotrichum candidum) to determine the extent of protein enrichment. According to the findings, the protein content of cassava mash fermented by a mixed culture of bacteria had the highest value (14.60 mg/g dry matter). Chikezie and Ojiako recently investigated the role of palm oil in conjugation with the duration of fermentation on gari cyanide and aflatoxin loads. The 48-hour fermentation scheme of processed cassava gari reduced cyanide content significantly, whereas the 48-hour fermentation with palm oil increased aflatoxin content reduction.

Enriched poultry feed or animal feed

Cassava peels, which are mostly generated as waste during cassava processing, have been used as an important source of starch in livestock feeds. Aside from carbohydrate content, cassava peels have a high level of crude fibre, which has facilitated its use as a potential source for animal feeds. Cassava peels, on the other hand, contain a high concentration of toxic cyanogens. Purwadaria reported that the use of mould in the SSF process is beneficial in the detoxification and degradation of toxic cyanogenic compounds. Furthermore, the fibre content of cassava peels decreases due to mould degradation activity and is thus used for poultry feed. The SSF process was carried out using cassava peels and wheat flour, as well as indigenous microbes, Aspergillus Niger or Panus tigrinus as inoculums. The fermented products demonstrated optimal substitution in poultry feed.

Enzymes in dairy processing

Milk and milk products contain a large number of enzymes, which are either naturally occurring or produced by microorganisms. Proteinases, lipase, lactase, catalase, xanthine oxidase, lactoperoxidase, and other enzymes are commonly found in dairy products. These enzymes are vital not only in preserving milk's natural values and producing desirable products when used wisely, but they also cause spoilage in dairy products. Some of these enzymes have traditionally been used in the production of various dairy products. Rennet, a generic term for commercial preparations containing acid proteases, is a well-known dairy enzyme used in cheese production. To meet the demands of the cheese industry, various other proteinases from plant, animal, and microbial sources have been tried to replace conventional rennet/chymosin. Recombinant chymosin, which is similar to calf rennet and superior to other rennet substitutes, has also been developed. In addition to milk-clotting enzymes used in the production of various cheeses, the dairy industry employs enzymes such as lipases, lactase, lysozyme, and lactoperoxidase for flavour enhancement, lactose hydrolysis, accelerated cheese ripening, and microbial spoilage control. One of the most promising applications of enzymes is the cleaning of dairy equipment, the removal of milk stones, and the formation of biofilms, which provide an alternative to conventionally available detergents and other cleaning agents.

Rennet and its substitutes

Cheese is defined as a product or substance formed by the coagulation of certain mammals' milk by rennet or similar enzymes in the presence of lactic acid produced by added or adventitious microorganisms, from which a portion of the moisture has been removed by cutting, warming,

and/or pressing, and which has been shaped in mould and then ripened by holding for some time at suitable temperature and humidity. Various types of cheese are produced all over the world, with rennet being an essential component in the process. Rennet is a saline extract extracted from the abomasums of young (less than 30 days old) milk-fed calves. However, the term can also refer to a substitute preparation if a name modifier is used (e.g., pepsin rennet, bovine rennet, microbial rennet, fungal rennet, etc.). When the term'rennet' is used in its broadest sense, it refers to calf rennet. The main enzyme in calf rennet or standard rennet is chymosin (rennin). It is commonly used as a milk-clotting agent in the production of high-quality cheeses with good flavour and texture. Due to an increase in global cheese production over the last three decades, combined with a decrease in calf rennet supply, a search for rennet substitutes has begun. Various rennet substitutes, including vegetable, plant, animal, and microbial sources, have been tried, and some of them are commercially availableAmong these, microbial rennet has gained widespread acceptance.

Rennin acts on milk protein in two stages, enzymatic and non-enzymatic, resulting in milk coagulation. Because of the influence of calcium ions and the temperature used in the process, the resulting milk gels during the enzymatic phase. Many microorganisms have been found to produce rennet-like proteinases that can be used in place of calf rennet. Microorganisms such as Rhizomucor pusillus, *Rhizomucor miehei*, Cryphonectria parasitica, and Aspergillus oryzae are widely used in cheese production to produce rennet. One major disadvantage of using microbial rennet in cheese production is the development of off-flavor and bitter taste in both nonripened and ripened cheeses.

Recombinant rennet6

Rennets derived from microbial sources seem to be more proteolytic in nature than rennet derived from animal sources, resulting in the production of some bitter peptides during the cheese ripening process. As a result, efforts have been made to clone the calf chymosin gene and express it in various bacteria, yeasts, and moulds.The FDA has also given recombinant chymosin GRAS status and approved its use in cheese manufacturing. Many different laboratories have cloned the calf prochymosin gene in Escherichia coli and studied the gene structure as well as the properties of the recombinant chymosin. In E. coli, the expressed proenzyme is mostly found as insoluble inclusion bodies made up of reduced prochymosin and molecules linked by disulphide bridges. Centrifugation is used to collect inclusion bodies after the cells have disintegrated. Individual laboratories reported slight variations in the procedure for renaturation of prochymosin from inclusion bodies, but all followed the same general scheme. Recombinant E. coli yeast has enzymatic properties that are indistinguishable from native calf chymosin.

The prochymosin gene has also been cloned in *Saccharomyces cerevisiae*, with levels of expression ranging from 0.5 to 2.0% of total yeast protein. In yeast, approximately 20% of the prochymosin is released in soluble form, which can be directly activated; the remaining 80% is still associated with cell debris. The prosequence is required for proper polypeptide chain folding in prochymosin. As a result, recombinant chymosin produced solely from the chymosin gene sequence (open reading frame) is unable to clot milk.

The zymogen for aspartic acid when compared to yeast, filamentous fungi secrete significantly more proteins into the culture medium. Furthermore, filamentous fungi secrete heterologous proteins with correct polypeptide chain folding and sulphydryl group pairing. A. oryzae has expressed the *R. miehei* protease gene. Kluyveromyces lactis, A. nidulans, A. niger, and Trichoderma reesei have all been found to express prochymosin. The reported yields of the model systems ranged between 10 and 40 mg of enzyme per litre of culture medium in the majority of cases. However, 3.3 g of enzyme per litre was obtained as well. Kluyveromyces lactis yeast has been used as an efficient host for the secretion of recombinant chymosin, resulting in a large-scale chymosin production process. If produced over an industrial scale, the yields could be significant. The majority of companies manufacture recombinant rennet of cattle calf origin in various microbial hosts. However, in India, the main source of milk is buffalo, which has a different composition than cows.

Buffalo rennet is naturally compatible with clotting buffalo milk (Mohanty et al., 2003). The National Dairy Research Institute in Karnal, India, has taken the lead in cloning the buffalo chymosin gene. The buffalo chymosin cDNA has been cloned in E. coli, and its sequence shows that it is highly homologous to cattle chymosin. Because the majority of the rennet added to cheese milk (> 90%) is lost in the whey, immobilisation would significantly extend its catalytic life, but its efficiency as a milk coagulant has been called into question. As a result, there is widespread agreement that immobilised enzymes cannot properly coagulate milk due to the inaccessibility of the Phe-Met peptide bond of k-casein, and that the apparent coagulating activity of immobilised rennets is due to leaching of the enzyme from the support.On an experimental or pilot scale, various types of conventional cheeses have been successfully made using recombinant rennet. There were no significant differences in cheese yield, texture, smell, taste, or ripening between cheeses made with recombinant chymosins and natural enzymes. According to biochemical and genetic evidence, recombinant chymosins are identical to calf rennet.

Enzymes in accelerated cheese ripening

Cheese ripening is a complex process mediated by biochemical and biophysical changes that transforms a bland curd into a mature cheese with distinct flavour, texture, and aroma. The desirable characteristics are produced by the partial and gradual breakdown of carbohydrates, lipids, and proteins during ripening, which is mediated by a number of agents, including I residual coagulants, (ii) starter bacteria and their enzymes, (iii) nonstarter bacteria and their enzymes, (iv) indigenous milk enzymes, particularly proteinases, and (v) secondary microflora and their enzymes. Proteinases used in cheese processing include (i) plasmin, (ii) rennet, and (iii) starter and nonstarter bacteria proteinases (cell wall and/or intracellular).

Approximately 6% of the rennet added to cheese milk remains in the curd after manufacturing and significantly contributes to proteolysis during ripening. Individual neutral proteinases and microbial peptidases in combination enhance cheese flavour and, when combined with microbial rennets, reduce the intensity of bitterness caused by the latter. Acid proteases isolated from microbial sources are extremely bitter. Various animal or microbial lipases impart the distinctive cheese flavour, low bitterness, and high rancidity, whereas lipases in combination with

proteinases and/or peptidases bestow good cheese flavour with low bitterness. Attenuated starter cells or Cell-Free Extracts (CFE) are preferred in a more balanced approach to accelerating cheese ripening using mixtures of proteinases and peptidases.Cheese ripening is primarily an enzymatic process that can be sped up by increasing the activity of key enzymes. When compared to using elevated temperatures, which can result in accelerating undesirable nonspecific reactions and, as a result, offflavor development, this has the advantage of initiating more specific action for flavour development. Enzymes can be added to cheese to develop specific flavours, such as lipase for the development of Parmesan or Blue-type cheese flavours. Because the pathways that lead to the formation of flavour compounds are largely unknown, the use of exogenous enzymes to accelerate ripening is largely an empirical process.

CHAPTER 10
LACTASE (β-GALACTOSIDASE)

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Lactases are hydrolases that catalyse the terminal residue of -lactopiranosil (Galb1-4Glc) to produce glucose and galactose. Lactose is found exclusively in dairy products in the human diet. Lactose is hydrolyzed into glucose and galactose by the intestinal -galactosidase (lactase) before absorption. In the body, these monosaccharides are absorbed and used as energy sources. Lactose maldigestion occurs when people with impaired lactose metabolism are unable to utilise lactose in milk. Lactase is a digestive enzyme that hydrolyzes lactose to improve the solubility and sweetness of dairy products.Lactose hydrolysis enables lactose-intolerant people to consume milk and other dairy products. Lactose, a sugar found in milk and whey, and its corresponding hydrolase (lactase or -galactosidase) have been studied extensively over the last decade. The enzyme immobilisation technique has opened up new and exciting possibilities for the use of this sugar.

Lactase enzyme is abundant in fermented milk produced by lactic acid bacteria (Lactococcus, Lactobacillus, Streptococcus species, and others). Yogurt bacteria produce lactase, an enzyme that hydrolyzes 20-30% of lactose to absorbable monosaccharides. This expression may also help people with lactose intolerance tolerate lactose in yoghurt rather than milk. Lactase is used by ice cream, yoghurt, and frozen dessert manufacturers to improve scoop and creaminess, sweetness, and digestibility, as well as to reduce sandiness caused by lactose crystallisation in concentrated preparations. Cheese made from hydrolyzed milk ripens more quickly than cheese made from regular milk. The cheese-making industry generates a large amount of whey as a byproduct, with lactose accounting for 70-75 percent of the whey solids. Lactase's hydrolysis of lactose converts whey into more useful food ingredients.

Lactases have also been used in the processing of dairy waste and as a digestive aid taken in tablet form by humans when consuming dairy products. Lactase can be derived from plants, animal organs, bacteria, yeasts (intracellular enzyme), or moulds. Some of these sources are used in the production of commercial enzymes. Lactase preparations derived from Aspergillus niger, Aspergillus oryzae, and Kluyveromyces lactis are considered safe. The most studied E. coli lactase is not used in food processing due to cost and safety concerns. Lactase properties (optimal temperature and pH) from various microorganisms determine their applications. For acid whey hydrolysis, fungal lactases with a pH range of 2.5-4.5 are used. In neutral pH 6-7.5, yeast and bacterial lactase are suitable for milk and sweet whey hydrolysis. The inhibition of enzyme activity by the product galactose is another important property determining the enzyme's application.

The enzyme, which is more susceptible to inhibition by galactose, is only active in an immobilised column in a dilute solution of whey (at low lactose level).

Enzymes in Meat Industry

Many countries, particularly Japan, Australia, New Zealand, and the developed countries of Europe and the Americas, have large meat industries. In the United States, for example, the retail equivalent value of meat was $85 billion in 2012. One-third of agricultural land (14 billion hectares) worldwide is used to grow animal feed (one billion tonnes). While demand for meat is increasing rapidly in developing countries such as China and Africa, it is largely saturated in many developed countries such as Western Europe and North America. However, the demand for high-quality meat remains significant in both developed and developing countries. Enzymes frequently play a significant role in increasing efficiency and lowering costs in the production of high-quality meat.

Enzymes are used in the meat industry for four specific purposes: meat tenderization, cross-linking meats, flavour development, and nutrition improvement. Many of these processes require a specific pH, temperature, and/or moisture level. The required enzymes are produced by microorganisms at relatively optimal temperatures or pH conditions in which these development occurs. However, genetic engineering is frequently required to optimise enzyme activities. Furthermore, using another host to produce the desired proteins reduces production time and simplifies the purification process in situations where the enzyme is difficult to obtain due to resource constraints.

Proteases used in meat tenderization, for example, require proteases found in fruits such as pineapples and kiwis. However, extracting enzymes from these fruits is a waste of food resources and time sensitive—harvesting the enzymes requires the fruit to be in production season. In contrast, bacteria require only 12-24 hours to produce enzymes, whereas fruits require months to produce proteases. Furthermore, bacterial enzyme production can occur at any time of year. Purification of the enzyme is also more efficient and less onerous when bacteria are used, as a fusion tagged protein engineered to bind to a specific matrix is used.The structure of meat is described below, followed by different types of meat processing and the enzymes used in these methods, with a focus on recombinant enzymes. The chapter concludes with a synopsis of the regulations governing the use of recombinant enzymes in food processing.

Muscle to Meat

Muscle in animals is fibrous because it contracts and expands to provide tensile strength.As a result, even after extensive tenderization, the meat retains its natural fibrous texture. Biochemical changes in the muscle begin soon only after animal is slaughtered. After death, the fibres continue to metabolise, and the ATP level is maintained via polysaccharide glycogen degradation and anaerobic glycolysis. This results in a decrease in pH from 7.2 to 5.5 as well as a decrease in temperature, which perpetuates a gradual decrease in ATP (Kemp and Parr, 2008). The main contractile protein complexes, which are normally separated in live animals, irreversibly bind together, resulting in rigour mortis, a macroscopic change.

Proteases are then used to degrade and tenderise the muscle proteins, shearing the two main components that comprise the meat's fibrous texture—myofibrils and connective tissue. Myofibrils are the fundamental units of muscle fibres composed of myosin and actin filaments. Collagen is the main component of connective tissue.

Changes in these components have a significant impact on the texture and water-holding capacity of meat. Following the slaughter of the animals, a series of enzymatic reactions occur within the meat. Calpains and cathepsins are two enzymes that are specifically active in the process. Calpains are calcium-dependent, non-lysosomal cysteine proteases (proteolytic enzymes), whereas cathepsins are lysosomal proteases that are activated by low pH in lysosomes. Both types of enzymes are found in mammals and many other organisms. Caspases, also known as cysteineaspartic proteases or cysteine-dependent aspartate-directed proteases, are another type of enzyme. They are required for apoptosis (programmed cell death), necrosis, and inflammation. Kemp and Parr investigated the use of human recombinant caspase 3 (rC3) expressed in Escherichia coli to weaken myofibrillar proteins in porcine muscle post mortem. The recombinant protein was created by inserting a full-length human caspase 3 cDNA insert into a vector, which had a polyhistidine tag at the C-terminus. E. coli was activated with 0.2 mM isopropyl-beta-D-thiogalactopyranoside before being purified using a liquid chromatography system. Many proteins, including alpha-actin, troponin T, myosin, myofibrillar proteins, desmin, and troponin, were found to be cleavable by the recombinant caspase.

Meat Tenderization

The texture and flavour of meat are important indicators of its quality. Myofibril integrity and connective tissue robustness are the two main factors that contribute to tenderness. Proteolytic enzymes are widely used to improve meat tenderness (Koohmaraie and Geesink, 2006). Bromelain, a pineapple proteolytic enzyme mixture, is used to tenderise tough meat due to its natural ability to hydrolyze fibrous proteins and connective tissue (Amid and Arshad, 2015). The three methods for introducing proteolytic enzymes into meat are dipping the meat in a proteolytic enzyme solution, pumping the enzyme solution into major blood vessels, and rehydrating freeze-dried meat. These three methods have been shown to be less than ideal because they can result in uneven tenderization and surface overtenderization. The most efficient way to introduce the protease solution is to inject the enzyme preparation into the animal prior to slaughter, which aids in even tenderization.

Tenderization is commonly evaluated using Warner-Bratzler shear and sensory evaluation, while the enzyme's mode of action is either myofibrillar or collagen proteins. Aspergillus oryzae proteases are among the most active proteolytic enzymes for meat. However, these enzymes rarely penetrate the meat. A combined treatment of protease enzymes from *Bacillus subtilis* and Aspergillus oryzae shows better penetration and a greater ameliorating effect on meat tenderness than either alone. Both species preferentially degrade myofibrillar proteins over collagen proteins, resulting in improved sensory outcomes.

Meat tenderization requires optimization in order to reduce the toughness from myofibrillar proteins while not over-tenderizing the meat. The two enzymes commonly used for

tenderization, bromelain and papain, have broad substrate specificities. As a result, peptides linked to bitter tastes may be produced, resulting in an off flavour. YaB, a microbial elastase from Bacillus sp., was studied and modified to improve its activity for meat tenderization. Subtilisin YaB normally acts on tyrosine and phenylalaine, but it has been modified by replacing amino acids Gly124 and Gly159 with alanine—an amino acid found in connective tissue proteins. The engineered YaB was more active than natural proteases like bromelain and papain, and it preferred elastin and collagen over myofibrillar proteins. Furthermore, an elastase from Bacillus sp. EL31410 not only recognises collagen and elastin in connective tissue, but it is also active at lower pH and at both low temperatures where the meat is typically stored and high temperatures when the meat is cooked. Proteases with optimal activity at low temperatures will be discovered and applied to meat tenderization as cold-adapted microorganisms are discovered and exploited.

Elastase from Pseudomonas aeruginosa, an opportunistic human pathogen, was another protease tested for meat tenderization. This elastase is much more efficient than other proteases at hydrolyzing insoluble elastin. However, P. aeruginosa's pathogenicity renders enzymes in its native host unsuitable for food use. As a result, Escherichia coli and Pseudomonas putidas have expressed the elastase gene lasB. However, the enzyme was discovered to be difficult to purify from these hosts, making commercial use unlikely. In contrast, the methylotrophic yeast Pichia pastoris was discovered to be an excellent host for elastase expression. Lin *et al.* (2009) discovered that modified and recombinant elastase extracted from P. aeruginosa and expressed in P. pastoris has extremely high activity, approximately 26-fold that of P. aeruginosa, and is extremely heat resistant.

Dry-cured Meats

Dry-cured meats are uncooked meat products made after eight to 24 months of ripening, during which an uncontrolled microbes proliferates on the meat's surface. The use of proteases can speed up the ripening process. On dry-cured meats, Penicillium aurantiogriseum has a high proteolytic activity. Penicillium chrysogenum has also been isolated and its enzymes studied from dry-cured meats.EPg222, the resulting enzyme, is extremely active against myofibrillar proteins, which are hydrolyzed during the drying stage. Collagen, the dominant protein in connective tissue, remained relatively unchanged. The Epg222 gene has been cloned and expressed in P. pastoris, and the recombinant protease has the same properties as the original host P. chrysogenum.

A lengthy ripening process is also required for the production of aromatic compounds from amino acids and fatty acids in cured meat. However, using only proteases and lipases will not produce the desired aroma or flavour because lipid oxidation and amino acid catabolism require different enzymes and occur at different rates. Aroma production necessitates the use of both types of enzymes. Purified proteinases (PrA and PrB) and aminopeptidases (Arginyl and Prolyl aminopeptidase) from Debaryomyces hansenii CECT 12487 can produce the desired sensory quality. These enzymes catalyse the hydrolysis of sarcoplasmic proteins, resulting in the production of ammonia, an increase in pH, and the acceleration of the proteolytic pathway.

Bacteria such as Lactobacillus curvatus, Acidophilus sakei, Pediococcusacidilactici, and Enterococcus faecalis are used to ferment sausages. Several recombinant enzymes from all these species have been used to boost fermentation processes. Lactobacillus sakei SR911's katA catalase gene was cloned and expressed in Lactobacillus plantarum TISTR850, a catalase-deficient strain. The recombinant strain had catalase activity that was approximately three times that of the natural strain and significantly reduced lipid oxidation. Lactic acid bacteria are typically required to produce the acid that lowers the pH of the meat in order to produce flavour and texture. When exposed to certain environmental conditions, such as an abundance of oxygen, the meat can quickly produce hydrogen peroxide and turn rancid. The hydrogen peroxide produced can react with iron during lipid oxidation and spoil the meat, especially if stored for an extended period of time. As a result, a high catalase activity in the recombinant strain can reduce lipid oxidation and meat spoilage.

Cross-linked Meats

Transglutaminases, which cross-link proteins, have been used to improve the texture, flavour, and shelf-life of meat products. Transglutaminase has the ability to adhere to the surface of meat and crosslink amines to glutamines in proteins via an acyl-transfer reaction. The bacterium Streptoverticillium mobaraense is primarily used in the industrial production of transglutaminase.

Because transglutaminase is active even in cold temperatures, it can bind raw meat during frozen meat storage. As a result, transglutaminase is commonly used in the storage of sausages, ham, and fish. Cross-linked meat is of interest because it takes inferior muscles, such as trimmings, and combines them to form whole meats that are more appealing to the consumer market. However, because of the nature of the processing used to create cross-linked meats, the products are frequently frozen and lose colour, making them more difficult to market.

Other oxidative enzymes, such as tyrosinases and laccases, have been investigated as potential candidates for cross-linking meat in addition to transglutaminase. None of these enzymes, however, are as of now used in the meat processing industry. The recombinant transglutaminase F XIIIa produced by *Saccharomyces cerevisiae* fermentation can restructure raw minced meat. This enzyme increased the cohesion, elasticity, and hardness of minced meat during a 90-minute treatment at 37°C. Salt and phosphate increased the effect of cohesion, exaggerating the results. The addition of F XIIIa, on the other hand, caused colour deterioration that was deemed undesirable. In contrast, a 23-hour treatment at 10°C did not result in significant changes to the meat. Future research attempting to reduce colour deterioration caused by F XIIIa would be extremely beneficial to consumers.

Fresh and Frozen Meat Storage

Fresh meat is susceptible to spoilage from a variety of bacteria, including those from the genus Pseudomonas, Acinetobacter, Lactobacillus, and a number of yeasts and moulds. The environment in which the meat is stored has an impact on the production of specific bacterial flora. In vacuum-packaged meat, for example, the growth of Lactobacillus curvatus and Lactobacillus sake is favoured, requiring glucose to be metabolised into lactic acid. Similarly,

leucine and valine are converted into isovaleric and isobutyric acids. These modifications produce a cheesy odour that dissipates once the package is opened. The spoilage caused by psychrotrophic Clostridium laramie also affects proteolysis. As a result of the reductive action of hydrogen sulphide on myoglobin, the meat produces a hydrogen sulphide odour, loses texture, accumulates liquid, and turns red.

Recombinant antifreeze proteins have been used to prevent ice crystallisation during freezing while having no effect on the freezing process itself. Reduced ice crystal formation results in less drip, less cellular damage, and less nutrient loss. As a result, the texture is preserved better. The use of the recombinant antifreeze protein rAFP in Lactococcus lactis has greatly increased the economic longevity of meat storage during all seasons.

Fish Meat

Unlike other meats, fish meat softens much faster than mammalian or poultry meat. As a result, maintaining firmness is a critical goal during the treatment and storage of fish meat. Natural tenderization and softening of fish meat are independent of rigour mortis, and matrix metalloproteinases and matrix serine proteinases have been implicated in auxiliary tenderization. The cross-linking enzyme transglutaminase has been used in the production of various fish meat to maintain and/or increase firmness. Natural transglutaminases from fish, on the other hand, have had limited success when expressed in microorganisms. A transglutaminase extracted from S. mobaraense is currently used commercially to stimulate gel formation and works independently of calcium.

While extracellular transglutaminases have advantages, the recombinant enzyme expressed by Streptomyces lividans and E. coli was less productive than the original strain of S. mobaraense. The recombinant enzyme was purified using cation-exchange chromatography and gel filtration, which was far less difficult than obtaining the enzyme from the original strain. Furthermore, the recombinant transglutaminase exhibited poor thermal stability and cross-linking activity, making it an unsuitable candidate for commercial production. If these properties can be improved, the expression of the enzyme in a different host would be ideal for producing a high quantity and purity of protein.

Surimi, a fish mince obtained by chopping, detendoning, and leaching of the fish skeletal muscle, is one specialty fish dish. Surimi can form a firm structure in the presence of salt by cross-linking unfolded actomyosin. Surimi's unique characteristics include gel-forming, water-binding, and oil-binding abilities, making it a valuable base component for a variety of food products. The hydrolysis of endogenous proteinases in several types of fish impedes the gel-forming process in surimi. Gel softening can be inhibited by recombinant chicken cystatin, a protease inhibitor.

E. coli has successfully produced recombinant cystatin, but whether it is safe for human consumption is unknown. The same enzyme has been successfully expressed in Pichia pastoris and then glycosylated to improve freezing stability, thermal stability, and pH stability. Centrifugation and column chromatography can be used to purify recombinant cystatin. Similarly, while the properties of cystatin expressed in yeast P. pastoris are similar to the wild type, the safety of human consumption requires further investigation.

Animal Feed

A major component of animal feed, is used to give poultry its distinctive yellow colour, which is caused by xanthophylls found in alfalfa. However, alfalfa is less desirable for providing high fibre content in meat, which affects the meat's quality and nutritional value. Many experiments have been carried out to improve animal feed, with varying degrees of success. Ponte et al., for example, attempted to improve the fibre content of the feed by combining cellulase from Cellvibrio mixtus with the recombinant xylanse GH11-CBM6 from Clostridium thermocellum. The final results showed that not only did the recombinant enzymes not contribute to the nutrition of the poultry, but they were individually harmful, causing a negative impact on final weight gain and feed intake. They also did not improve the efficiency with which feed was converted into supplements for the animals.

Meat Safety

The safety of meat-processing enzyme preparations is determined using scientific evidence and is evaluated under the conditions in which it is used directly or indirectly. The manufacturer must provide correct info about the product's composition, manufacturing method, usage, dietary exposure, toxicology, and other characteristics. In addition, the enzyme source must be non-pathogenic and non-toxigenic.

Before it can be released into the market, the enzyme is submitted to regulatory agencies such as the FDA in the United States for review and approval. During the approval process, the FDA frequently consults with the Food Safety and Inspection Service (FSIS), which evaluates the suitability of the enzyme preparation to ensure that the bare minimum of enzyme is used to produce the desired effect in meat.Transglutaminase is currently permitted in certain meats if the enzyme is used to bind and reform meat cuts. Labeling indicating that the product has been reformed is required. Furthermore, Aspergillus value Aspergillus niger, *Bacillus subtilis*, and *Bacillus subtilis* var. amyloliquefaciens protease preparations have been approved for use as meat tenderizers.

General Regulations on Recombinant Proteins Used in Food Processing

The regulations and policies governing enzyme preparations used in the processing of meat and other foods and beverages vary greatly between countries. The increasing use of recombinant enzymatic reactions and Genetically Modified Microorganisms (GMM) has contributed to this heterogeneity, as different regions have differing views on pre-market safety assessment of recombinant enzymes, ranging from thorough evaluations to none at all. Food additives are enzymes that are present in the final food product as an ingredient, usually for colour, taste, and texture, and processing aids or enzymes that are used during the food manufacturing process to catalyse reactions but are either removed or inactivated in the final product. However, commercial use approval may be contingent on the specific enzyme categorization system used in the country in question. For example, in the United States, Canada, and Japan, all food-processing enzymes are regulated as food additives, whereas they are considered processing aids in New Zealand and Australia.

Processing aids are used at very low concentrations during food production and are removed or deactivated during baking/cooking. The level of consumer exposure to residual enzymes in the food product is an important aspect of their safety assessment. The amount of Organic Carbon Solids (TOS) present in the concentrated enzyme prior to formulation is used to calculate this: The enzyme and other organic materials derived from the source microbial cultures are included in TOS (Olempska-Beer et al., 2006). The final enzyme preparation's safety is then assessed based on whether it meets the Food Chemicals Codex standards and specifications for enzyme preparations (FCC). FCC is a collection of internationally recognised standards for food ingredient purity and identity. The US Pharmacopeial Convention revises and publishes it every two years in the United States, and it includes a section on food-processing enzymes. FCC is recognised globally by regulatory agencies, manufacturers, vendors, and consumers. The regulatory frameworks for enzyme preparations around the world are briefly outlined in the following sections.

European Union (EU)

Food enzymes are currently regulated in the EU by Regulation (EC) No. 1332/2008. Enzymes used in food preparation are recognised by the EU as processing aids that serve no technological purpose in the final product and are exempt from the requirement that they be listed as flavourings on the product label (EUR-LEX, 2008). The European Commission requires the producer of an enzyme to submit an application with all necessary data, such as its source and intended use, to the European Commission, where the European Food Safety Authority conducts safety assessments (EFSA). Once an enzyme has been declared safe for its intended use by EFSA, it is entered into the Public Register of all food enzymes in accordance with Regulation EC 1331/2008. EFSA, on the other hand, requires that the data submitted by the producer in support of the application include the necessary information to verify that the enzyme has a technological use and that the intended use does not mislead the consumer. If residual enzymes are present in the final product, EFSA still approves it if they do not react with the food.

Article 12 of Regulation (EC) No. 1829/2003 requires GMO labelling on products containing ingredients derived from genetically modified organisms. However, recombinant processing aids are not included because they are only used during the manufacturing process and are not present in the final product. As a result, food articles made with recombinant effectively improve are not labelled as GMO.

CHAPTER 11

ENZYMES USED IN FOOD AND VEGETABLES INDUSTRY

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Fruit and vegetable juices are among the world's most popular liquids, with orange, apple, tomato, and grape juices among the most popular. Although the exact steps vary, all juices are generally made by mechanically pressing or macerating fresh vegetables and fruits in the absence of heat or solvents to extract their naturally occurring liquids. Enzymes capable of degrading plant cell-wall components are used in commercial juice production to facilitate extraction and increase juice yield. For genetically modifying these enzymes to optimise the juice manufacturing process, recombinant gene technology is now the most popular option. Manufacturers frequently concentrate the juice by removing approximately two-thirds of the water content, significantly increasing its shelf-lifeWater and lost flavours are reintroduced into concentrated juices before they are released to the market, or they are sold as bulk concentrated juice preparations to which consumers add water before consumption.

Pectinase

Pectinases, also known as pectinolytic enzymes, are the most common enzymes used in juice production. They catalyse the degradation of pectin molecules found in plant cells' primary cell wall and middle lamella. Pectins are complex glycosidic macromolecules composed primarily of galacturonic acid residues that contribute to the strength of cell walls by promoting the group cohesiveness of other cell wall components.Polymethylgalacturonases (PMG) catalyse the hydrolysis of -1,4-glycosidic bonds in the pectin backbone, whereas polygalacturonase (PGase) catalyses the hydrolysis of the same bond in polygalacturonic acid. Deesterifying enzymes such as pectin methyl esterase (PME), pectin lyases (PL) that randomly cleave glycosidic bonds in pectin, and pectatelyases (PGL) that selectively cleave glycosidic linkages in polygalacturonic acid are also members of the pectinase family. Pectinase preparations used in juice production are typically a blend of PGases, PGLs, and pectin esterases.

Use in Juice Extraction

The pectinase mixture is applied to the fruit pulp during juice extraction to degrade insoluble pectins in the cell walls that obstruct the free flow of the juice. As a result, the viscosity of the juice decreases, reducing pressing time and increasing juice yield (Heerd et al., 2012). When compared to traditional extraction methods, such as mechanical crushing and heating the release juices through plasmolysis of plant cells, the use of pectinase mixtures has resulted in over 90% juice yields from mashed puree (Lien and Man, 2010). Pectinolytic enzymes are naturally produced by a variety of plants, microbes, insects, and nematodes during plant infection, decomposition of dead plant material, and so on. Microbial sources, on the other hand, are used

exclusively in commercial production due to their low cost and ease of genetic manipulation, which allows for optimization of enzyme production and functionality.

The filamentous fungus Aspergillus niger, which has GRAS (Generally Regarded As Safe) status from the United States Food and Drug Administration (FDA), is the most commonly used to produce industrial pectinase preparations via submerged fermentation (SmF). Traditionally, fungal strains are cultured under various physical conditions and substrates to produce pectinase mixtures of varying compositions. However, the presence of undesirable substances in the food product may be caused by the activity of some pectinases in the mixture. For example, the activity of pectin esterases during wine production results in the formation of methanol, which is toxic to humans. Furthermore, even under ideal conditions, wild-type strains do not overproduce proteins in sufficient quantities for industrial use. With the development of recombinant technology, it is now possible to genetically modify bacteria to produce pectinolytic enzymes tailored to specific needs.

Heterologous Expression of Pectinases

Heterologous expression of eukaryotic pectinases in a prokaryotic host is a promising route to mass production of a single enzyme. Recombinant enzymes from various sources can be mixed in desired proportions to produce pectinase preparations free of unwanted substances and tailored to the needs of various applications.Due to its high yield of recombinant proteins and a well-studied annotated genome, Escherichia coli is the most widely used prokaryotic host for heterologous expression of both bacterial and fungal pectinases. Despite the fact that E. coli is unable to perform post-transcriptional and post-translational protein modifications, the recombinant pectinases it produces are generally functional. Indeed, recombinant E. coli strains have been used to successfully express and purify PGases, PMEs, PLs, and other pectinolytic enzymes.

Overexpression of recombinant proteins in E. coli, on the other hand, promotes the formation of insoluble protein aggregates known as Inclusion Bodies (IB), which impedes the recovery of active proteins. To recover the bioactive form, the partially denatured proteins in IBs must be re-solubilized and refolded in vitro, a process that can be prohibitively expensive for commercial purposes. Efforts to improve the recovery process have revealed that mild solubilisation conditions (Singh and Panda, 2005), culture incubation at room temperature rather than 37°C (Damak et al., 2013), and the use of smart polymers and affinity precipitation could all improve the recovery of active pectinases from IBs.The use of eukaryotic hosts for heterologous expression of eukaryotic proteins is an appealing prospect because they can perform post-translational modifications and produce large amounts of recombinant proteins.

Saccharomyces cerevisiae, a yeast with GRAS status and one of the best-studied organisms, has been successfully used as a host for recombinant PGases, PLs, and other pectinolytic enzymes from fungal, bacterial, and yeast origins. Because the vast majority of *S. cerevisiae* strains lack natural pectinolytic activity, they are ideal for overexpression and purification of a single recombinant pectinase. A. niger RH5344 endo-PGase was cloned and expressed in *S. cerevisiae*, and the resulting recombinant protein was more thermostable than wild-type PGase (Lang and

Looman, 1995). Because of plasmid continuity and increased plasmid copy number, the yield of the heterologous protein was also significantly higher. Similarly, an acidic PGase (PG1) from Aspergillus kawachii (IFO 4308) (Rojas et al., 2011), a pectatelyase (PelE) and a PGase from Erwinia chrysanthemi and a PME from A.

Aculeatus have been successfully expressed in *S. cerevisiae*. Several oenological *S. cerevisiae* strains expressing recombinant pectinolytic enzymes have also been developed as alternatives to the fungal pectinases currently used in wine filtration and clarification. A *S. cerevisiae* strain engineered to express a PGase (PG1) demonstrated faster wine filtration than traditionally used strains without changing wine composition, a strain engineered to express a PGase of yeast origin improved wine extraction yield compared to industrial strains (Fernandez-Gonzalez et al., 2005), and recombinant *S. cerevisiae* strains producing Pichia pastoris is another yeast that has been widely used as a heterologous host and has achieved remarkably high levels of constitutive expression of recombinant pectinases. *Kluyveromyces marxianus* CECT1043 PGase (EPG1-2) was cloned into P. pastoris, and the recombinant strain produced 200-fold more enzyme than the wild-strain. The glycosylation patterns of the protein in the eukaryotic host usually differ from those of the native protein: in some cases, the change in glycosylation patterns has conferred favourable characteristics on the recombinant protein (Sieiro et al., 2012). When a PGase (PG7FN) from the thermophilic fungus Thielavia arenaria XZ7 was expressed in P. pastoris, the recombinant PG7FN had a higher functional temperature (60°C), better pH stability, and higher catalytic efficiency.

Bi-functional Pectinolytic Enzymes

The discovery of natural bifunctional pectinolytic enzymes that contain two catalytic domains with different lipase activity within the last decade has raised the prospect of a significant advancement in juice/wine extraction processes in the near future. A gene from the genome of the alkaliphilic gram-positive bacterium Bacillus KSM-P358 with both PL and PME catalytic regions was isolated and heterologously expressed in *Bacillus subtilis* (Kobayashi et al., 2003). The recombinant protein was most active in the two catalytic regions at pH 8.5-10 and temperatures ranging from 40°C to 45°C. When the catalytic domains' gene sequences were separately expressed in E. coli, the functionality and catalytic properties were identical to that of the intact enzyme, implying that the bi-functional enzyme could function as a substitute for the two distinct catalytic functions (Kobayashi et al., 2003). A bi-functional amylase with PL and PME activity was isolated from the marine bacterium Pseudoalteromonas haloplanktis ANT/505, which grows at low temperatures ranging from 0 to 29°C (Truong and Schweder, 2006). The cold-adapted protein demonstrated high PME activity over a wide temperature range of 5°C-30°C at pH 7.5. Both of these enzymes are alkaline, as is typical of bacterial pectinases. S6A, a multimodular pectinase with an N-terminal PME catalytic domain and a C-terminal PGase domain, was recently discovered in the fungus Penicillium oxalicum (Tu et al., 2014a). The recombinant S6A showed both PGase and PME activity when cloned into P. pastoris, with the PME specific activity (271.1 U/ mg) being higher than that of most fungal PMEs tested for citrus pectin degradation.

The optimum temperature and pH were 50°C and 5.0, respectively, while pH 3.5-6 and 40°C maintained excellent stability. The majority of metal ions tested (including Na+, K+, Ca2+, Li+, Co2+, and Cr3+) increased PME activity without interfering with PGase functionality (Tu et al., 2014a). If the production and functionality of these bifunctional pectinases could be improved through further genetic engineering, they would be invaluable in commercial juice and wine production because they are cost-effective, environmentally friendly, and highly efficient.

Amylases

Amylases are enzymes that catalyse the hydrolysis of glycosidic linkages in starch in order to break them into manageable units. Amylases are widely used for starch degradation in industries such as food, textile, detergent, paper, and many more because starch is a common component of many biomaterials in human consumption. Amylases are used in the juice industry to clarify hazy, cloudy juices and produce clear juices.Starch, like pectin, contributes significantly to the cloudiness and turbidity of juice extracts, which impedes filtration, causes gelling when concentrated, and causes post-concentration haze As a result, de-pectinization and destarching are both required in industrial juice production. Amylases are classified into two types based on the type of glycosidic bond they hydrolyze. Endoamylases cleave -1,4-glycosidic bonds in the amylose (starch polymer) chain: -amylase, which is used in juice clarification, falls into this category (El-fallal et al., 2012). Exoamylases, such as -amylase, which is used in the detergent but instead textile industries, cleave the starch polymers' external -1,4-glycosidic bonds.

Natural Microbial Sources of Amylases

Amylases are found in the vast majority of living organisms, but microbial sources are used exclusively for industrial amylase production due to their low cost and ease of genetic manipulation. The majority of microbial strains used in industry are derived from a small number of well-studied bacterial and fungal species that naturally produce high levels of the enzyme. For example, the bacterial sources are primarily Bacillus spp., such as *B. subtilis*, B. amyloliquefaciens, and *B. licheniformis*, which can produce high levels of thermostable -amylases. The major fungal counterparts are A. niger, A. oryzae (Aunstrup, 1979), and Penicillium expansum.

Amylases are added to the juice extract after de-pectinization to produce clear juices, with the incubation temperature typically kept at around 50°C (Dey et al., 2014). Untreated juice extracts are acidic, with apple juice typically having a pH of 3.5 and lemon juice having a pH of 2.2-2.8. Despite the fact that Aspergillus spp. -amylases dominate industrial applications, their low thermal and acid stability pose a significant challenge to improving the juice production process. As a result, most research efforts have focused on developing amylases with high thermostability and functionality at low pH.

Furthermore, most wild-type -amylases discovered to date have a calcium (Ca2+)-binding premises: the enzyme is only fully functional when bound to Ca2+ (El-fallal et al., 2012). Ca2+-independent -amylases are highly desirable because this requires the addition of Ca2+ salts to the juice extracts, which must then be removed.

Heterologous Expression in Prokaryotic and Eukaryotic Hosts

Heterologous expression of -amylases in various prokaryotic and eukaryotic hosts has been successful in driving overexpression of the recombinant enzyme. The B. amyloliquefaciens -amylase gene was cloned into *B. subtilis*, and the recombinant enzyme production was 2500-fold higher than wild-type *B. subtilis* and five-fold higher than the donor B. amyloliquefaciens (Palva, 1982). The recombinant protein retained its functionality, and nearly the entire enzyme produced was secreted into the culture medium for easy purification. More recently, the -amylase encoding gene amy1 from a strain of *B. licheniformis* isolated from a starch farm was cloned and expressed in *B. subtilis* WB800, yielding a 1.48-fold increase in productivity compared to the donor strain. One disadvantage of using *B. subtilis* as a heterologous host is that it secretes a variety of proteins into the culture medium, including many proteases capable of degrading the recombinant -amylase protein.

To develop protease-deficient *B. subtilis* strains, various molecular techniques, such as deletion of protease genes and replacement of wildtype protease genes with mutants, have been used. A novel approach was used to determine the effect of increasing the number of translocons that transport proteins across the cell membrane on the amount of proteins secreted into the culture medium. In *B. subtilis*, an artificial operon containing the secYEG gene, the heterotrimeric protein complex required for protein translocation, was fused to an inducible promoter. The increase in secYEG protein levels was accompanied by a significant increase in -amylase secretion in the recombinants. This strategy can be used in the commercial production of recombinant -amylases to avoid jamming the cell membranes due to a lack of translocons, a phenomenon that frequently occurs with protein overexpression.

Several gram-negative bacteria, including E. coli and Pseudomonas fluorescens Biovar I, have also been used as hosts for heterologous -amylase expression. However, because intracellular IB aggregates form, additional steps are required to purify the recombinant proteins from these hosts. The most common eukaryotic hosts used in commercial -amylase production are Aspergillus spp., Pichia pastoris, and *S. cerevisiae* strains. In most cases, wild-type strains of Aspergillus spp. have been genetically modified to improve recombinant protein yield and functional stability. A. oryzae mutants with double deletions of CreA and CreB genes involved in carbon catabolite repression increased -amylase secretion tenfold. A. oryzae strains have also been engineered to contain more copies of the -amylase gene. While P. pastoris does not produce starch-degrading amylases naturally, it is widely used for heterologous expression due to its efficient protein production and secretory systems. -amylase from A. niger was functionally stable when cloned into P. pastoris at an industrial temperature of 50°C and a pH range of 3-6. (Zeng et al., 2011). An acid and heat-stable -amylase from Rhizopus oryzae was successfully cloned into P. pastoris and expressed under methanol induction (Li et al., 2011). Gene amplification has been attempted in an attempt to increase -amylase production in industrial strains. The -amylase gene from *B. licheniformis* (BLA) was amplified by homologs and expressed in the homologous host *B. licheniformis* B0204. When compared to wild-type B0204, recombinants with two to five copies of the gene expressed significantly more -amylase. In heterologous expression, the amylase gene is typically fused to a molecular tag, such as a poly-

histidine (His) tag, to facilitate affinity chromatography purification of the recombinant protein. For example, His-tag fusion at the C-terminus of *Bacillus subtilis* CN7 -amylase increased its turnover rate by 59%.

Recombinant Amylases with Unique Characteristics

Amylases with desirable properties isolated from unusual sources, such as extremophiles, are heterologously expressed to make production economically feasible. Heat and acid-stable, Ca2+-independent -amylases are highly desirable in the juice industry due to their stability under a variety of conditions, longer shelf-life, and potential for cost savings. Thermostable -amylase genes found in thermophilic microbes are frequently cloned and overexpressed in industrial bacterial strains such as *B. subtilis* and E. coli.

A highly heat-tolerant -amylase gene isolated from the hyperthermophilic archaeon Pyrococcus furiosus was cloned into *B. subtilis* and E. coli, where the recombinant protein demonstrated optimum activity at pH 4.5 and nearly 100°C. Other thermostable amylases isolated from various microbial sources have also been overexpressed in heterologous hosts with success (Emtenani et al., 2015; Grzybowska et al., 2004; Haki and Rakshit, 2003; Mehta and Satyanarayana, 2013). A Ca2+-independent -amylase from the acidophilic B. acidicola was cloned and expressed in E. coli BL21, where it demonstrated high thermostability (30°C-100°C) and acid stability (pH 3-7). (Sharma and Satyanarayana, 2012). Several other thermophilic bacteria Ca2+-independent -amylases have been identified and characterised (Atsbha et al., 2015; Malhotra et al., 2000; Sajedi et al., 2005; Singh et al., 2015). Attempts to synthesise Ca2+-independent -amylases have been limited to enzymes discovered in nature. The Ca2+-binding site of B-amylase was altered using site-directed mutagenesis**.**To reduce its reliance on Ca2+ for optimal function (Priyadharshini and Gunasekaran, 2007). When the mutant genes were cloned as well as expressed in E. coli, mutant amylase N104D demonstrated significantly higher specific activity at pH 5 and 70°C than the wild-type enzyme. Efforts to improve -amylases for use in the juice industry are ongoing.

Cold-active Amylases

Interest in cold-active -amylases for use in juice production, as well as cold-active pectinases (discussed above), has grown due to their potential for preventing microbial contamination and lowering energy consumption. Amylases produced by psychrophilic microbes contain polypeptides with greater flexibility, making substrate accommodation easier at low temperatures.

The first coldactive -amylase to be successfully crystallised and its 3D structure resolved was Alteromonas haloplanctis -amylase. It was successfully cloned into the mesophilic E. coli, and when expressed, the recombinant protein retained the wild-type enzyme's psychrophilic functionality. When the amylase gene from the psychrotolerant fungus Geomyces pannorum was engineered into A. oryzae, it was the first time a cold-adapted -amylase was heterologously expressed in filamentous fungi. The recombinant -amylase was most active at 40°C, but it retained more than 20% of its maximum activity at temperatures ranging from 0 to 20°C. Because their natural hosts have adapted to produce offspring under extreme environmental

conditions, the production of cold-adapted enzymes frequently necessitates highly specific physical parameters. It is not economically feasible to recreate these conditions for advertising enzyme production. As a result, before being used in industrial juice extraction and other commercial applications, cold-adapted -amylase production in a mesophilic background must be optimised.

Halophiles as Heterologous Hosts

Because of their basic nutritional requirements and ability to grow in extreme salt conditions, moderately halophilic microbes, particularly those belonging to the genus Halomonas, have emerged as sensitive cell factories to be used as heterologous hosts for recombinant amylases (Frillingos et al., 2000). H. meridian and H. elongate were able to secrete the thermostable -amylase from *B. licheniformis* in addition to producing their own extracellular -amylases (amyH) (Coronado et al., 2000). P. woesei -amylase was heterologously expressed in H. elongata, where the recombinant protein had been comparable in thermostability and functionality to the wild-type protein.

Microbial Sources of Recombinant Cellulases

Commercial cellulases are primarily derived from filamentous fungi, specifically Aspergillus species and Trichoderma species. These species naturally secrete large amounts of cellulases into the extracellular environment, making them ideal hosts for heterologous expression of recombinant cellulases due to their efficient secretion system and higher specific growth. The protease-deficient A. niger D15 strain was used as the host to express several recombinant cellulases, yielding highly homogeneous recombinant proteins (Rose and Zyl, 2008). T. reesei strains were engineered to express the cbh1 gene, an important subunit of cellulase, under a strong promoter, resulting in significantly increased recombinant cellulase production and enhanced enzymatic activity.

E. coli, *B. subtilis*, and the yeast P. pastoris have all successfully expressed recombinant cellulases.Cellulases must be as thermostable and acid-stable as the other components of the enzyme cocktail used during juice production if the commercial juice extraction process is to be optimised. Thermomonospora fusca thermostable cellulase was expressed in E. coli, and the recombinant cellulase E3 was discovered to be active at temperatures above 60°C and to be more resistant to proteolysis. Similarly, high-yielding thermostable cellulases have been expressed in *B. subtilis*, the thermophilic fungus Talaromycesemer sonii, and the thermotolerant yeast Kluyveromyces marxianus NBRC1777.

Xylanases

The enzyme xylanase converts -1,4-xylan, the second most abundant component of the plant cell wall after cellulose, into xylose, facilitating the breakdown of the plant cell-wall matrix. One of its numerous commercial applications is in the extraction and clarification of juices and prosecco Commercial xylanases are mostly produced by Aspergillus spp., Trichoderma spp., and a few bacterial species like Bacillus spp. and Streptomyces spp. The use of recombinant DNA technology to improve xylanase production and desired characteristics is becoming more

common, and the number of commercially available recombinant xylanases is steadily increasing. To increase production rates, xylanases are heterologously expressed in both eukaryotic and prokaryotic hosts, including E. coli, P. pastoris, and T. reesei (de Faria et al., 2002). Codon optimization in P. pastoris resulted in high extracellular expression of a recombinant xylanase with appealing biochemical properties from Streptomyces sp. S38. In order to increase cell surface secretion, xylanase from A. niger was expressed in P. pastoris using a variety of secretion signal sequences. A single-copy PIR, which can be used in commercial xylanase production, produced the highest xylanase secretion.

Several thermophilic microbes' thermophilic xylanases have been isolated and cloned into heterologous hosts for over-expression. Thermomyces lanuginosus thermostable xylanase was highly efficiently produced in P. pastoris by expressing the gene under the AOX1 promoter (Damaso et al., 2003). The native protein's optimum temperature of 75°C was retained by the recombinant protein. Thermostable xylanases from deep-sea thermophilic GeoBacillus spp. MT-1 (Wu et al., 2006), Rhodothermus starting at age.

Naringinase

Citrus fruit juices, which include many popular fruits such as oranges, grapefruits, lemons, limes, and tangerines, have a distinctive bitter taste due to the presence of a molecule known as naringin. Citrus juices are frequently treated with naringinase, an enzyme capable of degrading naringin during production, to remove excessive bitterness. Nariginase immobilised on food contact—approved films or columns are widely used in the commercial sector to reduce the activation energy and Michaelis constant for the naringin hydrolysis reaction. Historically, filamentous fungi have been the primary microbial source of commercial naringinase. A. niger.

Marine Microbiological Enzymes

To meet the needs of the world's growing population, the food and beverage industries produce a diverse range of foods and beverages. Dairy and milk, grain and cereal, fruits and vegetables, beer and hard liquor, meat and poultry, seafood, packaged or convenience foods, and packaged drinks are among the products manufactured (Chandrasekaran et al., 2015). The use of enzymes in the food processing industryresults in superior products with higher yields, as well as a lower carbon footprint, energy consumption, and environmental pollution, as well as low greenhouse gas emissions and raw material waste. Modern enzyme technology's primary goals continue to be the preservation of foods and food components, the efficient use of raw materials, the improvement of food quality through texture and taste, the production of dietetic foods, the elimination of anti-nutritive substances from certain nutritional raw materials, the utilisation of raw materials for the preparation of animal feed, and process optimization to reduce processing costs. Several traditional chemical reactions have thus been partially or completely replaced by enzymatic methods, with enzymes being widely used in the food processing industry (Figure 11.1).

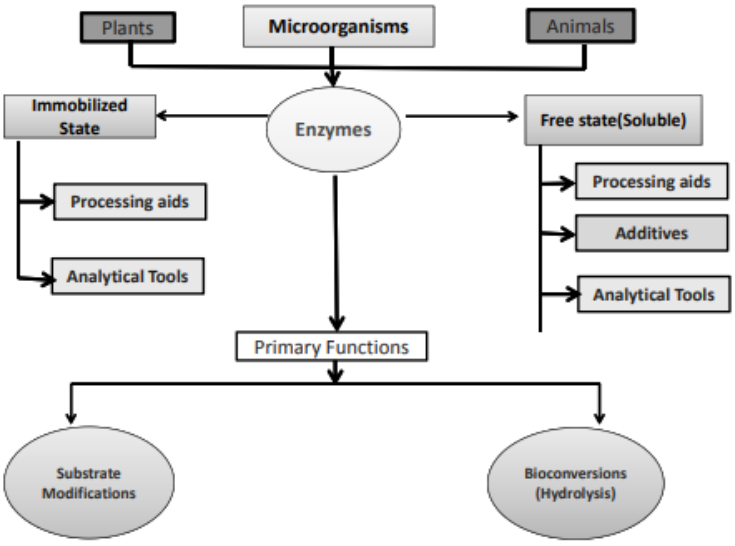

Figure 11.1 represent the enzymes and types of applications in food industries.

Marine Environment as Source of Food Grade Enzymes

The marine environment ranges from nutrient-rich to nutritionally sparse, with only a few organisms surviving. Because of the complexity of the marine environment, which includes high salinity, high pressure, low temperature, and special lighting conditions, enzymes produced by marine microorganisms differ significantly from homologous enzymes produced by terrestrial microorganisms. Furthermore, marine microorganisms have evolved to be extremely adaptable to a wide range of environmental conditions, including high salt concentration, extreme temperatures, acidic and alkaline pH, extreme barometric pressure, and low nutrient availability. They have evolved unique metabolic capabilities to ensure survival in a variety of habitats, and they produce a variety of metabolites that are not found in terrestrial microbial products (Faulkner, 2000). Marine microorganisms participate actively in the mineralization of complex organic matter in the marine environment via degradative pathways of their metabolism, contribute to secondary production in the sea, and are regarded as important ecological components in the marine environment due to their performance in biogeochemical cycles.

Microbes live in a variety of marine habitats such as neuston, plankton, nekton, seston, and epibiotic, endobiotic, pelagic, and benthic environments. Archaebacteria, cyanobacteria, eubacteria, actinomycetes, yeasts, fi lamentous fungi, microalgae, algae, and protozoa are all found in these environments. Almost all of these groups are unexplored potential sources of useful enzymes.Furthermore, in comparison to the terrestrial environment, the marine environment is home to marine microorganisms with distinct genetic structures and life habitats (Stach et al., 2003). Marine microorganisms are gaining popularity as a source of new enzymes because microbial enzymes are more stable than corresponding enzymes that come from living things.

Furthermore, marine microbial enzymes are of particular interest due to their superior stability, activity, and tolerance to extreme conditions that most other proteins are incapable of

withstanding. These characteristics are frequently useful in industrial processes.Bacteria and fungi in marine environments secrete various enzymes depending on their habitat and ecological functions. So far, most of the interesting marine enzymes with distinct structure, novel chemical properties, and biocatalytic activity have been identified as coming from archaea, extremophiles, and symbiotic microorganisms. However, they have yet to be fully utilised for commercial food applications.

Food Grade Marine Microbial Enzymes

Because of their habitat-related differences, marine microbial enzymes are of particular interest. Surprisingly, the enzymes found in marine environments belong to one or more of the main groups of enzymes, which include oxidoreductase, transferases, hydrolases, lyases, isomerases, and ligases. Enzymes produced by marine bacteria and fungi include protease, amylase, alginate lyases, chitinase, cellulase, ligninase, pectinase, xylanase, lipases, proteases, glutaminase, asparaginase, arylsulphatase, phosphatase, beta lactamase, and nucleus.

Lipase (EC 3.1.1.3)/esterases (EC 3.1.1.X)

Esterases (EC 3.1.1.X) and lipases (EC 3.1.1.X) are carboxyl hydrolases that cleave to form ester bonds. Esterases and lipases are widely used in the food industry to improve the quality of bread through changes in flour lipids; to enhance the flavour of butter, cheese, and margarine; to produce crackers and pasta; to degumming vegetable oils; to increase the titer of polyunsaturated fatty acids in vegetable oils; and to improve the digestibility of natural lipids (Visser et al., 2012; Ferreira-Dias et al., 2013). Because of their distinct catalytic properties in reactions such as esterification, hydrolysis, and trans-esterification of lglycerols in the production of food emulsifiers, lipases are important in the food processing industry. The marine Vibrio sp. VB-5 produces a lipase (EC 3.1.1.3) that hydrolyzes fish oil containing n-3 polyunsaturated fatty acids (PUFA). Lipase easily liberated saturated and monoenoic fatty acids from fish oil. Extracellular lipase was found in the marine fungus Aspergillus awamori BTMFW032 (Soorej et al., 2011) and A. sydowii (Bindiya and Ramana, 2012). Lipase production was also observed in marine Bacillus smithii BTMS 11 separated from marine sediments.

Protease6

Proteases are used in the production of cheese, baking, meat tenderization, and the hydrolysis of soy protein. Microbial proteases are used in brewing, meat processing, and as a digestive aid, among other applications. Proteases are enzymes that break down peptide bonds in proteins and polypeptides.al They are used in a variety of processes in the food industry as relatively crude preparations under a variety of operational conditions (temperature, pH, osmolarity) and may or may not require high specificity in enzyme action.As digestive enzymes, several proteases are used. A protease derived from the marine yeast Metschnikowia reukaufii W6b has been reported to have applications in the cheese, food, and fermentation industries. In the skimmed milk clotting test, it demonstrated high skimmed milk coagulability. Some of these processes may employ proteases isolated from marine sources. It was discovered that several marine bacteria produce proteases. Protease was produced by halophilic *Bacillus licheniformis*, V. gazogenes, and Vibrio sp. 5709 from marine sediments.

Subtilisin (EC 3.4.21.62)-like serine protease from the psychrophilic marine Vibrio sp. PA-44, neutral protease BBP-127 from Bacillus sp. JT0127, metal neutral proteinases from the marine luminescent Vibrio harveyi and Vibrio splendidus, and a novel thiol protease hydrolyzing benzoyl-arginyl-4-methyl.An alkaline protease isolated from the marine Aureobasidium pullulans HN2- 3 was cloned and expressed in Yarrowia lipolytica, where it was used to produce bioactive peptides for nutraceuticals. In the food industry, aminopeptidases isolated from a marine Aspergillus flavus are used for debittering and enhancing the functional properties of protein-based products, as well as for cheese flavouring. Within the spectrum of extra-cellular enzymes from heterotrophic microorganisms in an Arctic fjord, psychrophilic leucyl amino peptidases with chymotrypsin and trypsin activities have also been identified.

Agarase

Agarases are used in the hydrolysis of agar, a seaweed-derived colloid that is commonly used as an additive in food processing because it can act as an emulsifying, gelling, and stabilising agent. Agar is a compound composed of agarose and agaropectin. Agarose can be hydrolyzed to produce oligosaccharides, which are particularly useful in the production of functional foods (Ramnani et al., 2012). Hydrolysis can be promoted by either α-agarases (EC 3.2.1.158), which cleave α-1,3 linkages to produce agaro oligosaccharides 3,6-anhydro-L-galactose (Anr) residues at their reducing ends; or by β-agarases (EC 3.2.1.81), which cleave β-1,4 linkage to produce neo-agaro oligosaccharides with D-galactose residues at their reducing ends (Fu and Kim, 2010; Chi et al., 2012). Agarases can exhibit either endo- or exo-activity, or both (Chi et al., 2012). Most agarases identified to date are -agarases, with -agarase isolated only from Alteromonas agarlyticus GJ1B (Potin et al., 1993), Thalassomonas sp. JAMB-A33 (Ohta et al., 2005), and Pseudoalteromonas sp.

Alteromonas sp., Bacillus cereus, Cytophaga sp., Pseudoalteromonas sp., Pseudomonas sp., and Vibrio sp. have all been isolated from marine sources. The gram-negative bacterium Microbulbifer maritimus was also found to produce an extracellular agarase of 75.2 kDa. Alteromonas GNUM-1 was used to isolate -agarase. Pseudomonas stutzeri, Aeromonas sp., and Vibrio sp. isolated from sea produced agarase and agarase-0107, an endo-type -agarase that hydrolyzed the -1, 4-linkage of agarose to yield neoagarotetraose and neoagarobiose at the a pH of around 8.

CHAPTER 12

BASICS OF INULINASES

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Inulinases are enzymes that have hydrolytic activity over inulin, a polyfructan found in several plants that contains fructosyl units linked together by (2, 1) linkages. Total or partial hydrolysis of inulin results in the formation of fructose or fructooligosaccharides. These are commonly used in the food industry, either as a sweetener or as a prebiotic. Endo-inulinases (EC 3.2.1.7) and exo-inulinases (EC 3.2.1.80) are distinguished according to whether they cleave the inulin chain into smaller oligosaccharides or hydrolyze the terminal fructose from the inulin chain (Basso et al., 2010). Extracellular inulinase from the marine Pichia guilliermondii OUC1 could be used to make ultra-high fructose syrups, whereas inulinase from the marine Yarrowia lipolytica OUC2 could be used to make inulooligosaccharides. Marinimicrobium sp. LS-A18 produced extracellular inulinase that was alkali-tolerant (Li et al., 2012). An extracellular inulinase from Nocardiopsis sp. DN-K15 demonstrated alkali tolerance (pH optimum of 8.0, and activity within pH 5.0-11.0) and thermostability (optimum 60°C). Both enzymes showed exo-inulinase activity, indicating that they could be used to make ultra-high fructose syrups. Bacillus cereus MU-31 generated exoinulinase extracellularly.

Chitinases

Chitosan is approved for use as a food additive or dietary supplement in Japan, England, the United States, Italy, Portugal, and Finland. Chitosan was approved as a functional food ingredient by Japan's health department in 1992. Japan and the United States are the two largest producers of chitosan, with smaller operations in India, Italy, and Poland. Chitinase has been isolated from a variety of marine bacteria, including Alteromonas, Pseudomonas, Moraxella, Acinetobacter, Vibrio, and Vibrio ischeri species. A solid-state fermentation (SSF) was developed for the production of extracellular chitinase using chitinous solid waste from the shellfish processing industry as a solid substrate and Beauveria sp. isolated from marine sediment as the enzyme producer.

When exposed to various types of chitin, Vibrio harveyii excreted several chitin-degrading proteins into the culture medium, allowing for efficient utilisation of various forms of chitin and chitin by-products. Chitinases C1 and C3, as well as chitobiose-deacetylase, are produced by Vibrio alginolyticus H-8. Aeromonas hydophila H-2330 secreted chitinase only when chitin was present as a C-source, indicating that chitinase is an inducible enzyme. Alteromonas sp. N-acetylglucosamine-deacetylase (EC 3.5.1.33) is a drug and a biocatalyst in the production of M o, which is useful in the production of glucosaminooligosaccharides with antimicrobial and anti-tumor activities. Vibrio sp. P2K-5 produces N-acetylglucosamine-6-phosphate-deacetylase (EC 3.5.1.25).

Extremozymes (Enzymes from Extremophiles)

Hot and cold streams, acidic and alkaline water flow, bright surface, dark deep sea with high barometric pressure, hyper-saline pyrolytic vents or cold seeps enriched with different minerals/gases, and deep sea volcanoes are all part of the marine ecosystem. These environments support a diverse range of flora and fauna in the water and on the seafloor, as well as extremely diverse microbial populations, including several extremophiles. Extremophiles are the primary source of extremozymes, which are active in extreme environmental conditions. Because archaea -amylase, pullulanase, and -glucosidase are active in the same pH and temperature range, they could be used in a one-step process for industrial bioconversion of starch.

Thermostable Enzymes

Extremozymes are considered the most important from an industrial standpoint because higher removal efficiency, lower viscosity, better mixing, faster reaction rate, and lower risk of microbial contamination at high temperatures facilitate many industrial processes. For example, thermostable enzymes isolated from thermophilic marine microorganisms, such as a thermostable a-amylase (Fuelzyme1) isolated from a deep sea bacterium by Verenium1 Corporation (Verenium-Fuelzyme1, 2012), were found to be active over a wide temperature range and increased fuel ethanol yields due to improved starch hydrolysis at much lower concentrations.Deep-sea thermal vent thermophilic microbes and hyperthermophilic bacteria that grow at 80-100°C are potential sources of highly specific and extremely thermostable enzymes. Thermostable polysaccharolytic enzymes are commonly used in the food processing industry to convert starch into oligosaccharides, cyclodextrins, maltose, or glucose, whereas acid-stable enzymes may improve starch liquefaction. Extremozyme has increased the efficiency with which cyclodextrins are produced from cornflour in commercial use. Cyclodextrins aid in the stabilisation of volatile substances (such as flavourings in foods), the improvement of medicine absorption by the body, the reduction of bitterness, and the masking of unpleasant odours in foods and medicines.

Thermostable marine microbial enzymes have mostly been reported from thermophilic Thermotoga, Thermus, Thermococcus, Pyrococcus, Bacillus, and Sulfolobus members. Under anaerobic conditions, Pyrococcus abyssi, a deep-sea hyperthermophilic archaebacterium, produces thermostable -glucosidase (I) (EC 3.2.1.21), which will be useful in the cellulose industry and the production of sugar derivatives. This species is also capable of producing thermostable esterase. Pyrococcus sp. KOD1 is another hyperthermophilic Pyrococcus sp. that produces a thermostable thiol protease.

Cold Adapted Enzymes

The oceans, which have an average temperature of 1° to 3° C (34 to 38° F), cover more than half of the Earth's surface. The vast land areas of the Arctic and Antarctic are either permanently frozen or only unfrozen for a few weeks in the summer. Microbial communities thrive in Antarctic ice-ocean water, which remains frozen for the majority of the year. Photosynthetic eukarya, particularly algae and diatoms, as well as a variety of bacteria, make up these communities. These cold-loving organisms have attracted manufacturers who require

refrigerator-temperature enzymes, such as food processors (whose products often require cold temperatures to avoid spoilage). Cold-adapted enzymes have been found in abundance in deep sea, cold streams, cold seeps, and polar seas. Cold-adapted enzymes catalyse reactions at low temperatures, reducing energy consumption while also stabilising thermolabile reactants/products, minimising evaporation, and reducing the possibility of microbial contamination.

Furthermore, cold-adapted enzymes have much higher specific activities than their mesophilic counterparts. MCP-01, an abundant extracellular serine protease produced by the deep-sea psychrophilic Pseudoalteromonas sp. SM9913, was active down to 0°C but deactivated at 40°C due to autolysis. The latter prevents over-tenderization during and after cooking.This collagenase was effective in tenderising beef meat at 4°C, as it reduced the beef meat shear force by up to 23%, and it was as effective as bromelain, a widely used meat tenderizer. Myroides profundi D25, another collagenase from the deep sea, was used to further elucidate the mechanism of collagen degradation. A pseudoalterin secreted from deep-sea sediment by" has become synonymous sp. CF6-2 has been reported to be used in elastin degradation.

Alteromonas sp. produced a novel psychrostable metallo protease (almelysin). Extracellular hydrolases such as protease, chitinase, glucanase, esterase, lipase, phospholipase, and DNA-degrading enzymes have been isolated from marine bacteria isolated from various locations in the permanently cold Arctic and Antarctic habitats. A-amylase from Alteromonas haloplanctis, isocitrate dehydrogenase and lipase from Vibrio sp., lactamase triose phosphate isomerase from Moraxella sp., and subtilisin from Bacillus sp. are some of the cold-adapted enzymes from bacteria. The potential use of psychrophilic xylanases derived from Antarctic microorganisms in the baking industry has been reported, and the enzymes have already been patented (Collins et al., 2006). Several cold-active lipases have been isolated from Antarctic deep-sea sediments and metagenomes, including Pseudomonas and Psychrobacter species.

Alkalophilic Enzymes

Several food processing reactions necessitate extreme acidic or alkaline conditions. Alkaline enzymes are enzymes that are energetic at highly alkaline pH and are produced primarily by alkalophiles, which have had a significant impact in industrial applications. The industrial production of cyclodextrin with alkaline cyclomaltodextrin-glucanotransferase is an important application (EC 2.4.1.19).

This enzyme reduced production costs and paved the way for its widespread use in foodstuffs. Alkaline phosphatase is an important alkaliphilic enzyme found in marine microorganisms (Plisova et al., 2005; Sebastian and Ammerman, 2009). Some alkaline enzymes known from marine environments include thermo stable alkaline phosphatase (EC 3.1.3.1) with a wide pH range and endo-1, 3-beta-D-glucanase (EC 3.2.1.39) from Alteromonas macleodii KMM162; heatlabile alkaline phosphatase from a Gram-negative Antarctic marine bacterium; alkaline metallo endopeptidase from a marine A marine Streptomyces was found to produce a novel alpha-amylase that is pH stable for 48 hours and retains approximately 50% of its activity at 85°C.

Halophilic and Halo Tolerant Enzymes

Some halobacteria produce exoenzymes that have optimal activity at high salinities and have the potential to be used in harsh industrial processes where concentrated salt solutions otherwise might inhibit many enzymatic conversions. Several extracellular enzymes from marine microorganisms perform best when exposed to high salt concentrations. These enzymes are used in industrial processes where the reaction mixture contains high salt concentrations, which inhibit most industrial enzymes. Halophilic proteolytic enzymes are useful in peptide synthesis and may find use in the fish and meat processing industries.

Halo-tolerant enzymes are uniquely adapted to function in low water availability, and many of them have been reported to be organic solvent tolerant (Marhuenda-Egea and Bonete, 2004). Amylases produced by Halobacterium halobium and H. sodomense, proteases produced by H. salinarium and H. halobium, and lipases produced by several halobacteria are examples of halobacterial exoenzymes.

Micrococcus varians subsp. halophilus nuclease H is used in the industrial production of flavouring agents 5'-guanylic acid and 5'-inosinic acid in a bioreactor system with a flocculated cell column. Several halotolerant bacteria produce sodium enzymes, such as amylase, which is stable at 60°C and 5M NaCl and could be used to treat effluents containing starchy or cellulosic residues.

Microbial Enzyme Production Methods

The enzyme industry relies heavily on bulk production of enzymes at reasonable costs, which is dependent on the development of feasible large-scale enzyme production with appropriate bioprocesses and economical downstream enzyme processing. Unfortunately, in the field of marine bacterial technology, such ventures are largely ignored. Commercial enzyme production is primarily accomplished through submerged fermentation (SmF) and SSF. SmF is the most common bioprocess used in the enzyme industry for commercial-scale production. Extracellular enzymes (proteases, amylase, cellulases, tannases, and lipases, for example) can be easily produced in large quantities by both bacteria and fungi under SSF, whereas intracellular enzymes with a wide range of applications are typically produced under SmF and subjected to extensive downstream processing.

However, the literature on marine microorganisms, particularly on enzyme fermentation, is scant and limited to a few reports. These include L-gutamianse production by Vibrio costicola under SSF, with polystyrene used as an inert support, L-glutaminase and chitinase production by Beauvaeria sp. under SSF, continuous production.Although several marine bacteria, yeasts, and cyanobacteria have been observed to produce economically important enzymes, studies on fermentation production of the same have not been attempted. Indeed, based on the available literature on enzyme production by marine microorganisms, it was discovered that these marine microorganisms require sea-water as the solvent in enzyme production, in addition to complex media containing a diverse range of nutrients that are ideally found in sea-water for their growth and enzyme production. This requirement is impractical for transferring technology to a commercial-scale operation.

Furthermore, downstream processing of enzymes from salt-rich cultivation media presents significant challenges for enzyme purification, and the volume of enzyme released by these organisms is also an important consideration when selecting a sea-water-based cultivation medium for enzyme production. Another major barrier in the transfer of technology to industries is the development of ideal bioreactors/fermenters for the cultivation of marine microorganisms and large-scale enzyme production.Nonetheless, there is considerable interest in exploring marine biodiversity in order to harness marine microorganisms with the potential for novel enzymes in a variety of applications, including food and beverage processing.

Potential Enzymes

Extremophiles are microorganisms that grow and survive in harsh environments. MacElroy coined the term "extremophile" for the first time in 1974. (MacElroy, 1974). Extreme environments, according to Madigan and Marrs (1997) and Rothschild and Manicinelli (2001), include those with high (55 to 121°C) and low (-2 to 20°C) temperatures, alkaline (pH > 8) and acidic (pH 4). Extremophiles are organisms that can withstand extreme conditions such as high pressure, high levels of radiation, or toxic compounds. The temperature, pressure, pH, salinity, and water activity limits at which life can thrive are still unknown. Extreme environment is a relative term; an environment that is extreme for some organisms' growth may be essential for others. The domain Archaea contains the majority of the known extremophiles. Many extremophiles, on the other hand, have been identified and classified as belonging to the bacterial, archaeal, and eukaryotic kingdoms.

Extremozymes are enzymes derived from extremophilic microbes that function in conditions thought to be too harsh for proteins. Such conditions are common in industrial processes, resulting in an increasing demand for extremozymes (Elleuche and Antranikian, 2013). Extremophilic microbes outperform their mesophilic counterparts in this regard, as they are a rich source of naturally tailored enzymes for use in extreme conditions. There are only a few commercially available extremozymes, such as DNA-polymerase from Thermus aquaticus and related organisms. Every type of extremophile has distinct characteristics that can be used to provide enzymes with a diverse range of applications (Adams et al., 1995; Yano and Poulos, 2003). Many novel enzymes have evolved as a result of advances in molecular biology and protein engineering, and their applications in industrial biotechnology are contributing to the growth of this multibillion-dollar commercial sector.

There are numerous advantages to using enzymes in food processing industries. Enzymes are highly specific in their action, and enzyme-catalyzed processes produce fewer by-products and side reactions, resulting in higher-quality products with less pollution. A number of companies are forming to produce enzymes. Denmark leads the world enzyme market, with Novozymes (45% of the market) and Danisco (17%). Other companies include Genencor (the United States), DSM (the Netherlands), and BASF (Germany).

Starch Industry

Anselme Payen, a French chemist, discovered the first enzyme, diastase, a starch-degrading enzyme, in 1833. One of the most abundant sources of energy is starch. This complex

polysaccharide is a common food additive and a source of various sugar syrups used in fermentation, pharmaceutical, and confectionary industries. It is a heterogeneous polysaccharide made up of glucose units linked by -1, 4 and -1, 6-glycosidic bonds, resulting in the insoluble polymers are long amylose and the soluble branched polymer amylopectin. Enzyme synergy is required for complete hydrolysis of starch to glucose. Enzymes involved in the hydrolysis of starch to sugar include -amylases, glucoamylases, -glucosidases, and pullulanases. These enzymes account for 25% of the global enzyme market and are found in a wide range of microorganisms, including extremophiles.

The saccharification of starch is a multistep process. The starch is first gelatinized in a jet cooker at 105°C for 5 minutes, followed by liquefaction (95°C for 1 hour at pH 6.0) of raw starch granules in the presence of bacterial -amylase. The next step is saccharification (60°C for three hours at pH 4.5), which is done in the presence of glucoamylase and pullulanase. The -amylase currently used in starch processing (derived from Bacillus a. flavus and *B. licheniformis*) is most active at 95oC and pH 6.8 and is Ca2+ stabilised. As a result, industrial processes involving these enzymes cannot take place at low pH, i.e., 3.2-4.5, which is the pH of native starch. The pH of the starch slurry is increased to 5.8-6.2 in order to be well-suited to the optimal pH of the enzyme required for liquefaction, and Ca2+ is supplemented to increase the activity and/or stability of the enzyme.

The second step, saccharification with glucoamylase, necessitates pH adjustment to 4.2-4.5 and salt removal, both of which take time and increase the process cost. As a result, enzymes that are ideal for this process must have a combination of properties, such as activity and stability at pH 3.0-4.0, 90-110°C, and Ca2+ independence.-Amylase is an endo-acting enzyme that cleaves -1, 4-linkages in starch and related substrates at random, resulting in branched and linear -anomeric oligo- and polysaccharides with varying chain lengths. Several -amylases have been identified from various extremophiles. -Amylase from Alicyclobacillus acidocaldarius was one of the first examples of a heat and acid-stable enzyme, with a pH 3.0 and 75°C optimum. There has been very little research on thermo-acid-stable -amylases. A. acidocaldarius and Bacillus acidicola thermo-acid-stable -amylases are useful in the starch industry. At 100°C, the -amylase from the hyperthermophilic archaeon Pyrococcus furiosus is most active. Another thermostable -amylase from Methanococcus jannaschii has been reported, with an optimum temperature of 120°C. Pyrococcus woesei -amylase is the initial crystallised enzyme from a hyperthermophilic archaea that grows best at temperatures above 100°C. The biophysical properties of thermophilic amylases

The structure of microorganisms is highly compact, with an increased number of salt bridges (Linden and Wilmanns, 2004). Ca2+-independent -amylases are those that do not require Ca2+ for activity or stability (Sharma and Satyanarayana, 2010; Rao and Satyanarayana, 2007). As evidenced by the preceding reports, -amylases from extremophiles with thermostability, acid stability, or Ca2+ independence have been discovered, paving the way for the development of an industrial enzyme with all of these properties in combination.Glucoamylases are exoenzymes that cleave -1, 4-glycosidic bonds in starch and other polysaccharides to release single -Dglucose units from non-reducing ends. This glucose can be used to make ethanol, amino acids, organic

acids, and other products in the food and beverage industries (Polakovic and Bryjak, 2004). In the presence of glucose isomerase, it is also converted into crystal dextrose and high glucose syrups, or isomerized to fructose. The majority of known thermophilic glucoamylases are from fungi, with only a few reports from bacteria and archaea. Aspergillus niger produced the first gene encoding glucoamylase. Thermoanaerobacter tengcongensis has the most thermostable glucoamylase from bacteria, which is optimally active on maltooligosaccharides with only four monosaccharide units at pH 5.0 and 75°C. Picrophilus torridus, P. oshimae, and Thermoplasma acidophilum were the first thermoacidophilic archaeal glucoamylases discovered. Archaeal glucoamylases have been found to be most active at pH 2.0 and 90°C (Serour and Antranikian, 2002), whereas glucoamylases produced by fungi, yeast, and bacteria are most active at 70oC and pH 3.5-6.0. A neutral glucoamylase from the thermophilic mould Thermomucor indicae-seudaticae that is most active at pH 7.0 and 60oC has been shown to be useful in starch saccharification.-Amylase, another exo-acting enzyme, attacks the starch's -1, 4-glucosidic linkage and produces the dimeric sugar -maltose. P. furiosus, a hyperthermophilic archeaon, has been found to produce -amylase, which is most active on pNP-maltopyranosid). Clostridium thermosulfurogenes produces a thermo-acid-stable -amylase that is most active at pH 5.5 and 75°C.

Endo-acting pullulanases hydrolyze -1, 6-glycosidic linkages in starch, pullulan, amylopectin, and related oligosaccharides. Amylopullunases are bifunctional enzymes that cleave both the -1, 4 and the -1, 6 linkages in starch, amylose, and other oligosaccharides, as well as the -1, 6 linkage in pullulan. This enzyme is used in the starch industry to produce sugar syrups such as maltose and maltooligosaccharides syrups, which are used in the food industry. Thermococcus kodakarensis KOD1 has been found to produce a thermostable pullulanase with a half-life of 45 minutes at 100°C.

Baking Industry

Bread is a popular and traditional food all over the world. The use of enzymes in baking has resulted in significant improvements in flavour, texture, dough flexibility, machinability, stability, loaf volume, crumb structure, and shelf-life. The baking industry accounts for nearly one-third of the total enzyme market for enzymes used in food applications. Baking enzymes are flour additives that act as dough conditioners. The baking industry primarily employs five types of enzymes: amylase, protease, xylanase, oxidase, and lipase. All of these enzymes work together to enhance the organoleptic properties of bread. Amylases used in the baking industry require properties that differ from those used in the starch industry. Baking necessitates the use of acid-stable and maltogenic amylases with intermediate thermostability. The enzyme's maltogenic nature has an antistaling effect on bread, while intermediate thermostability causes enzyme inactivation at the end of baking, preventing residual enzyme activity and product deterioration. Because the pH of the dough is acidic, -amylase's acid-stability is important in baking. Baking can benefit from an acidic -amylase from Bacillus acidicola that is most active at pH 4.5.

Xylanases were first used in the baking industry in 1970, and they are most commonly used in conjunction with amylases, lipases, and a variety of oxidoreductases to improve the rheological

properties of dough and the organoleptic properties of bread. These enzymes have also been used to improve the quality of baked goods such as cookies and cakes. The breakdown of hemicellulose in wheat flour and redistribution of water are important functions of xylanases in baking, leaving the dough soft and easy to knead. Its inclusion in dough aids in water absorption, resistance to fermentation, and bread volume increase.

Insoluble arabinoxylans that interfere with the formation of the gluten network are removed using xylanases. It produces high molecular weight solubilized arabinoxylans, which increase viscosity. As a result, a stable, flexible, and easily manageable dough is formed, resulting in increased loaf volume, soft crumb, better texture, and crumb structure (Rouau et al., 1994; Courtin and Delcour, 2001; Sorensen et al., 2004; Heldt-Hansen, 2006). Furthermore, the use of xylanases during dough processing is expected to increase the concentration of arabinoxylooligosaccharides in bread, which has beneficial effects on human health (Bhat, 2000). Collins et al., 2002a, 2003; Van Petegem et al., 2003) found that the GH family eight psychrophilic xylanase from Pseudoalteromonas haloplanktis increased loaf volume. Shah *et al.* (2006) used acidstable xylanases (optimum pH 5.3) from the acidophilic fungus Aspergillus foetidus as a bread improver in the production of whole wheat bread. Extremophiles with thermoacid-stable xylanases include Pyrococcus furiosus (102°C, pH 6.0), Sulfolobus solfataricus (105°C, pH 5.3), and Thermotoga neapolitana (102°C, pH 5.5). Recently, a thermo-alkali-stable xylanase from the polyextremophilic Bacillus halodurans was found to be useful in whole wheat bread.

Protease is used in baking to reduce mixing time, improve dough consistency, regulate gluten strength, and improve bread texture and flavour.In the bread industry, protease, a fungal acid, is used to modify gluten-containing mixtures. Because the blend contains proteases, it is soft and easy to pull and knead. These enzymes have a significant impact on dough rheology and bread quality, possibly due to their effects on the gluten network or gliadin (Salleh et al., 2006). They act on wheat flour proteins, reducing gluten elasticity. As a result, shrinkage of dough or paste after shaping and sheeting is reduced. Proteases are also used in the production of pastries, cookies, and biscuits. The hydrolysis of glutenin proteins in flour is responsible for dough elasticity and improves cookie spread ratio.The action of amylopullulanase on starch and other related polysaccharides results in the formation of maltotriose and maltooligosaccharide syrups, which are used in baking to prevent starch retrogradation and as an antistaling agent.

Fruit Juice Industry

Over the last five years, the global fruit and vegetable processing industries have seen consistent demand with an annual growth rate of 1.3%. Increased demand for processed foods, particularly in developing countries, is expected to boost industry revenue by 0.4% by 2020, from $271.3 billion to $273.1 billion.The most important steps in fruit juice processing are extraction, clarification, and stabilisation. Pectinases have traditionally been used prior to the extraction step to facilitate pressing and thus maximise juice yield. Generally, pressing is followed by a concentration step or a 'hot break' step at high temperatures, such as 90°C. Because commercial enzymes are not thermostable, the enzyme treatment requires the addition of enzymes from extremophiles.

All fruits that gel after crushing contain pectin. It keeps the juice in the mash, reduces pressability, decreases juice yield, and prevents clarification and evaporation for concentration. The primary goal of enzymatic treatments is to reduce pectin viscosity in order to accelerate processing. Pectin is a polysaccharide group with a large molecular structure, not a single substance. Pectin is made up of galacturonic acid residues in long chains. A carboxyl (-COOH) group is present on each residue, which is sometimes esterified by that of the addition of methyl groups (-COOCH3). Pectin that has had half or more of its acid residues methoxylated is known as high methoxyl pectin, while those that have had fewer are known as low methoxyl pectin.

In nature, nearly 80% of galacturonic acid carboxylic groups are esterified with methanol. There are three major types of pectinases based on their mode of action.Pectinesterase (PE), also known as pectinmethylhydrolase, catalyses the deesterification of pectin's methoxyl group to pectic acid. The second class of depolymerizing enzymes is divided into two subclasses: hydrolyzing enzymes like polymethylgalacturonase (PMG) and polygalacturonase (PG), and cleaving enzymes like polymethylgalacturonate (PMGL) and polygalacturonate lyase (PGL). PMG catalyses the hydrolysis of pectin's -1, 4-glycosidic bonds, particularly in highly esterified forms. The hydrolysis of -1, 4-glycosidic linkages in polygalacturonic (pectic) acid is catalysed by PG. PMGL catalyses the transeliminative cleavage of pectin, whereas PGL cleaves the -1, 4-glycosidic linkage in pectic acid. The protopectinase enzyme group converts protopectin (a parent pectic substance that produces pectin and pectinic acid upon restricted hydrolysis) to highly polymerized soluble pectin.

CHAPTER 13

FUNDAMENTALS OF ANIMAL FEEDS

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The use of enzymes in animal nutrition is important in today's farming systems. Exogenous enzymes have already been shown to improve nutrient utilisation and performance in poultry. The poultry industry consumes the most feed enzymes. The poultry industry's integrated nature has aided in the rapid adoption of new technologies and exogenous enzymes in the feed industry to improve nutrient digestibility and utilisation. More than 90% of broiler diets in the United Kingdom, Australia, New Zealand, and Canada contain feed enzymes, and nearly 70% of wheat and barley-based poultry feeds contain glycanases (xylanases and -glucanases). Feed enzymes improve nutrient digestibility and the degradation of undesirable components in feed that are harmful or of little or no value. Cereal-based diets, such as barley, rye, and wheat, contain high levels of non-starch polysaccharides (NSPs), which, when supplemented with NSP enzymes, may reduce intestinal methane production. Animal feed supplementation with enzymes like xylanases, amylases, cellulases, pectinases, phytases, and proteases reduces unwanted residues like phosphorus, nitrogen, copper, and zinc in the excreta, which helps to reduce environmental contamination. Xylanases are enzymes that are added to animal feed to hydrolyze arabinoxylans. Arabinoxylans are found in grain cell walls and have an anti-nutritional effect in poultry. Cellulases used in feed processing improve feed digestibility and animal performance. Bedford *et al.* used Trichoderma cellulases as a feed additive to improve the feed conversion ratio and/or increase the digestibility of a cereal-based feed.

In response to rising phosphorus (P) pollution from animal operations, microbial phytases are being widely used in poultry and pig diets. The European Union's ban on the use of meat and bone meal (major sources of P) increased the use of microbial phytases. As a result, the demand for microbial phytase as the primary feed enzyme has increased globally. Phytases degrade phytic acid (myo-inositol1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate), which is the most common form of P stored in plant-derived ingredients.Frost and Sullivan estimated that the global market for feed enzymes was around $344 million in 2007 and was expected to reach $727 million in 2015. Currently, commercially available feed enzymes by catalytic type include: 3-phytase, 6-phytase, subtilisin, -galactosidase, glucanase, xylanase, -amylase, and polygalacturonase. Microbial phytases are primarily used to improve mineral bioavailability and food processing in animal (swine and poultry) feeds and human foodstuffs. Fungi and yeast are known to produce xylanases and phytases. Recently, it was reported that phytase from the yeast Pichia anomala can efficiently reduce the phytic acid content of various broiler feeds. There have been few reports of acidophilic bacteria and archaea producing xylanases and phytases. Some reports of acidstable xylanases include the production of an enzyme from S. solfataricus that demonstrated activity on carboxymethyl cellulose at pH 3.5 and 95°C. Another xylanase report

comes from Acidobacterium capsulatum, which has optimal activity and stability in the acidic range (Inagaki et al., 1998).

When processing temperatures exceed 70°C, available exogenous enzymes become significantly less stable. To avoid thermostability issues at high pelleting temperatures, enzymes are added as liquids via post-pelleting application systems. Applying liquid enzymes precisely after pelleting can be a time-consuming and costly process. As a result, thermostable enzymes are required, which will simplify the application of dry product prior to pelleting and promote the use of the enzyme in pelleted diets.

There have been reports of thermo-acid-stable phytases from Pichia anomala and Sporotrichum thermophile. There have been no reports of acidophilic bacteria and archaea producing stable phytases. Acidic phytases from extremophilic bacteria and archaea are being researched for use in animal feeds because they are expected to have better thermo-acid stability, substrate specificity, and catalytic efficiency than fungal phytases.

Scale up and Enzymes

The production of bulk chemicals and enzymes has given SSF an advantage. Several researchers have demonstrated bulk enzyme production using SSF. Solid-state fermentation with the Aspergillus carbanerius strain on wheat bran medium produced pectolytic enzymes on a large scale. Fermentation for 21 hours at 30^{o}C in the tray resulted in maximum enzyme production.

Furthermore, steaming wheat bran at 15 Pa for 45 minutes increased enzyme production (Ghildyal et al., 1981). Roussos *et al.* described Zymotis, a novel large-scale solid-state fermenter that aids in upscaling studies with a capacity of 4-12 kg substrate dry matter (SDM) or 15-55 kg moist solid medium, depending on the initial moisture content of the medium. Sugarcane bagasse and wheat bran [80:20 (w/w)] were fermented to produce cellulase. The fermentation was carried out for 64 hours at 28^{o}C, with the medium aerated at a rate of 300:l humidified air/h/ compartment for the first 12 hours, and then doubled for the remainder of the fermentation time. The experiment compared the production of cellulolytic enzymes by Trichoderma harzianum in Zymotis to that of a medium-sized laboratory column fermenter. When compared to parallel fermentation in the laboratory-scale column fermenter, the enzyme titres in Zymotis were marginally higher at all substrates. Better control of based on culture parameters in the Zymotis is attributed to its improved performance.

The scaling-up of a bioprocess is a critical stage that ensures the economic viability of the bio-product in question. It consists in increasing the size of the bioreactor in order to achieve sufficient productivity, while maintaining as much as possible the outputs obtained during laboratory or pilot scale tests. Most biotechnological products (such as enzyme production) are only viable at bioreactor volumes of a few hundreds of thousands of litres. Several aspects are understood by the definition of the scale-up. It would not only be about increasing the size of the bioreactor, but also about the costs, outputs, and device simplicity. In the case of microbial fermentation for enzyme production, a broad multidisciplinary field is shared, and scaling up becomes an important link for bioprocess transfer from the laboratory scale to the industrial scale for commercial needs.

Scaling up occurs in stages: from Erlenmeyer flask to laboratory bioreactor, laboratory bioreactor to pilot bioreactor, and pilot bioreactor to industrial bioreactor. It is not possible to keep all of the parameters the same or proportional to the scaling. Each transfer to a larger scale is complicated because various parameters, such as the scale of sterilisation, aeration, and agitation, change when the volume, diameter, and height of the bioreactor are increased. Despite the increase in culture volume, similar physicochemical conditions must be maintained in the environment of each cell. Various parameters are analysed and modified at each stage of the scale up because the physicochemical and enzymatic reactions occurring inside the bioreactor vary depending on the volume of the bioreactor used. Furthermore, during scaling, it is critical to consider the investment costs of equipment (bioreactor, extraction and purification techniques) and operation (culture medium and energy); to automate equipment if possible; to reduce waste production; and to obtain products of the desired quality.

The modelling in SSF is the representation of the working system in terms of mathematical expression. The use of mathematical modelling techniques to describe biological and transport phenomena within the system improved understanding of how to design, operate, and scale-up SSF bioreactors significantly. Equations from bioreactor mathematical models describe various features of the conceptually divided micro level and macroscale phenomena that occur within SSF bioreactorsThe scale-up prediction models consider growth kinetics, rotating-drum, traditional and Zymotis packed-bed, sporadically forcefully-aerated, and well-mixed bioreactors. Globally developed aspects include growth kinetics, energy, and water balance, with some assumptions made to handle the models.

Cofactor

A cofactor is a molecule that is not a protein that aids a biological process. Cofactors can be useful chemicals that aren't generally found in amino acids, such as metal ions, organic compounds, or other molecules. While the body can create some cofactors, such as ATP, others must be received through diet. Understanding how biological processes take place at the molecular level involves cofactors in great detail. Whether cofactors are present or not can affect how quickly a reaction transitions from reactant to product.For biological studies of health, an understanding of cofactors is crucial. Without the required cofactors, either humans or animals may develop fatal diseases or pass away ultimately. A cofactor is a molecule that is not a protein that aids a biological process. Cofactors can be useful chemicals that aren't generally found in amino acids, such as metal ions, organic compounds, or other molecules. While the body can create some cofactors, such as ATP, others must be received through diet.Understanding how biological processes take place at the molecular level involves cofactors in great detail. Whether cofactors are present or not can affect how quickly a reaction transitions from reactant to product. For biological studies of health, an understanding of cofactors is crucial. Without the required cofactors, either humans or animals may develop fatal diseases or pass away ultimately.

Enzyme Nomenclature

Depending on the reaction they are used to catalyse, enzymes are divided into six main classes. All enzymes are given an "EC" number since the nomenclature was established by the Enzyme

Commission in 1961 (with the most recent revision taking place in 1992). The categorization does not take into consideration protein structure, chemical process, or homology of amino acid sequence.

Enzymes Classification

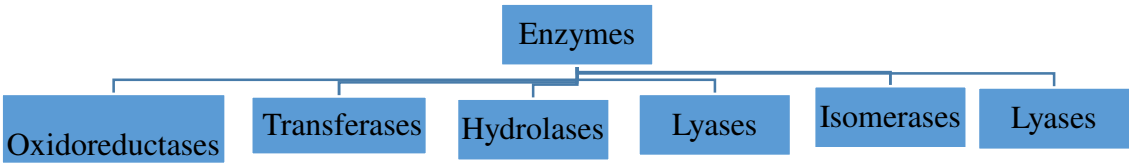

Figure 13.1: Illustrate the classification of enzymes.

In the past, enzymes were given names based on who discovered them. Further study led to a more thorough categorization.

1. Oxidoreductases: These serve to catalyze oxidation and reduction processes, such in the conversion of pyruvate to acetyl coenzyme A by pyruvate dehydrogenase.
2. Transferases: These aid in the chemical group transfer from one compound to another. A transaminase, which moves an amino group from one molecule to another, is an example.
3. Hydrolases: They help a bond to hydrolyze by catalysis. For instance, the pepsin enzyme breaks down protein peptide connections.
4. Lyases: These facilitate bond breaks without the need for catalysis, for as when the glycolysis enzyme aldolase splits fructose-1, 6-bisphosphate into glyceraldehyde-3-phosphate or dihydroxyacetone phosphate.
5. Isomerases: They aid in the compound's isomerization by catalysis. In the case of glycogenolysis, phosphoglucomutase catalyses the conversion of glucose-1-phosphate to glucose-6-phosphate (a phosphate group is moved from one place to another in the same substance) (glycogen is converted to glucose for energy to be released quickly).
6. Ligases: The association of two molecules is catalysed by ligases. By creating a phosphodiester link, DNA ligase, for instance, catalyses the joining of two segments of DNA.

Mechanism of enzyme action

In the majority of chemical reactions, an energy barrier must be broken in order for the reaction to take place. This barrier keeps intricate molecules like nucleic acids and protein from decaying on their own, which is essential for the survival of life. However, some of these complex molecules must be disassembled and this energy barrier must be overcome when metabolic changes are needed in a cell. The extra energy required (known as activation energy) may be provided by heat, but the cell would perish as a result of the temperature increase. The option is to utilise a catalyst to reduce the activation energy level. This is the function of enzymes. As they interact with the substrate, an intermediate complex is created, known as a "transition state,"

which takes less energy to complete the reaction. The enzyme remains unmodified and is free to interact with more substrate molecules as the unstable intermediate chemical swiftly degrades to produce reaction products

The enzyme's active site is the only area that can bind to the substrate. The protein's folding arrangement creates a pocket or groove known as the active site. The specificity of an enzyme is determined by its three-dimensional structure, together with the electrical and chemical characteristics of the cofactors and amino acids that make up its active site.Genetic regulation and distribution in a cell have an impact on both enzyme production and function. Some cells do not create specific enzymes, whereas other cells only produce particular enzymes when needed. The distribution of enzymes within a cell is not always uniform; frequently, they are compartmentalized in the nucleus, on the cell membrane, or in subcellular organelles. Neurosecretions, hormones, as well as other substances that impact the internal milieu of the cell also alter the rates of enzyme production and activity.

Factors Affecting Enzyme Activity

Only a very little amount of an enzyme is required to catalyse a reaction since they may be utilised again and are not destroyed in the processes they catalyse. 1,000 substrate molecules can be converted every second by a typical enzyme molecule. An enzymatic reaction's rate rises with increasing substrate concentration and reaches its peak when all of the enzyme molecules' active sites are occupied. The rate of the reaction is therefore considered to be saturated by the pace at which the active sites may change the substrate into the product. There are several methods for inhibiting enzyme function. When molecules that are very similar to the substrate molecules attach to the active site, they hinder the real substrate from binding. For instance, the competitive inhibitor penicillin prevents the activity of an enzyme that several bacteria utilize to build their cell walls.

When an inhibitor attaches to the enzyme somewhere other than the active site, noncompetitive inhibition takes place. Noncompetitive inhibition may occur when an inhibitor binds to an enzyme in a way that physically blocks the enzyme's typical active site. In some cases, it is thought that the binding of the inhibitor alters the shape of the enzyme molecule, causing the active site to become distorted and preventing the enzyme from interacting with its substrate. Allosteric inhibition is the name given to the latter form of noncompetitive inhibition, and the allosteric site is the location where the inhibitor binds to the enzyme. A metabolic pathway's final product frequently acts as an allosteric inhibitor on an earlier enzyme in the route. This negative feedback occurs when a byproduct of an enzyme's process inhibits it.

Both stimulation and inhibition of enzyme activity can occur under allosteric control. By altering its shape to match a substrate that couldn't effect the change on its own, an activator molecule can be coupled to an allosteric site and used to trigger a reaction at the active site. Hormones and the byproducts of previous enzymatic activities are common activators. The cell can produce energy and materials when they are required and can be inhibited from doing so when there is an enough supply due to allosteric stimulation or inhibition.In order to develop, heal damaged tissues, and get energy, biochemical processes are required, and they occur within the bodies of

all living things. In living things, these processes are known as metabolism, and they take place constantly. When they quit functioning, the organism perishes. All of the processes that take place in living things require a lot of activation energy to get started. A catalyst is used to speed up chemical processes and lower the activation of energy in order to minimise the amount of energy the cell uses.

Effect of Temperature

The enzymes are highly sensitive to heat changes since they are proteins. Unlike typical chemical processes, enzyme activity is temperature-dependent. Each enzyme has a certain temperature range where it is more active, as you have seen. The ideal temperature, which falls between 37 and 40 degrees Celsius, is at this point.When the temperature exceeds the ideal temperature, the enzyme activity steadily declines until it reaches a particular temperature, at which point it entirely ceases due to a change in the enzyme's natural makeup.On the other hand, if the temperature falls below the ideal level, the enzyme's activity decreases until it reaches the lowest temperature, when it is least active. At ^{0}C, enzyme activity ceases entirely; but, if the temperature increases again, the enzyme becomes reactivated.

Enzyme Function

1. Several variables, such as the following, have an impact on enzyme activity.
2. If there is a sufficient amount of substrate available, raising the concentration of enzyme will speed up the rate at which it reacts.

The substrate concentration:

The substrate concentration is the limiting factor when the enzyme concentration is constant and when the substrate concentration is decreased. The rate of the enzyme reaction accelerates with increasing substrate concentration. A larger concentration of substrate does not speed up the process since the enzymes get saturated with it at very high substrate concentrations.The climate: Each enzyme operates optimally at a certain temperature. Enzyme activity typically rises with increasing temperature. More molecular collisions occur as a result of increased molecular mobility as temperature rises. However, if the temperature exceeds a particular degree, the heat will denature the enzyme, ruining its three-dimensional form and causing its hydrogen bonds to become denaturized. By reducing molecular mobility, cold temperature, on the other side, slows down enzyme activity. Each enzyme has a perfect pH range that aids in maintaining its three-dimensional form. By modifying the charge of the enzyme, pH changes can denature enzymes. The enzyme's ionic bonds, which contribute to its functional shape, are altered as a result.

Salt concentration:

1. There is an ideal salt concentration for each enzyme. Enzymes may be denatured by changes in the concentration of salt.
2. Several interactions between bacteria, their enzymes, and how disinfectants and temperature extremes are used to manage bacteria.
3. Chlorine, iodophores, mercurials, formaldehyde, iodine, silver nitrate, and ethylene oxide are only a few disinfectants that inactivate bacterial enzymes and obstruct metabolism.

4. Proteins and enzymes are denatured during high-temperature processes including pasteurisation, boiling, and autoclaving.
5. Enzyme processes are slowed or stopped under cold temperatures, such as those associated with refrigeration and freezing.

Enzyme Kinetics

The study of enzyme kinetics examines the rates of chemical processes that they catalyse. In enzyme kinetics, the rate of the reaction is determined, and the implications of changing the reaction's parameters are explored. The catalytic mechanism of an enzyme, its function in metabolism, how its activity is regulated, and how a medication or modifier (inhibitor or activator) could influence the rate can all be learned through studying an enzyme's kinetics in this wa.Typically, an enzyme (E) is a protein molecule that catalyses the reaction of its substrate, another molecule (S). This forms an enzyme-substrate complex (ES) by binding to the enzyme's active site. From there, the complex undergoes transformation into an enzyme-product complex (EP), and from there to product P, via a transition state (ES*). The mechanism is the collection of actions:

$$E + S \rightleftarrows ES \rightleftarrows ES^* \rightleftarrows EP \rightleftarrows E + P$$

The simplest scenario of a reaction with a single substrate and product is assumed in this example. There are examples of this: triosephosphate isomerase is an enzyme that catalyses any one-substrate, one-product reaction, and mutases, such as phosphoglucomutase, catalyse the transfer of a phospho group from one position to another. The enzymes that catalyse two-substrate, two-product reactions, for instance the NAD-dependent dehydrogenases like alcohol dehydrogenase, that catalyses the oxidation of ethanol by NAD+, much outweigh these less prevalent enzymes.

Though less frequent, reactions involving three or four substrates or products are possible. There is no need that the number of products and substrates be equal; glyceraldehyde 3-phosphate dehydrogenase, for instance, has three substrates and two products. Enzyme kinetics can also display the order in which substrates bind or products are released when enzymes, like dihydrofolate reductase (shown to the right), bind numerous substrates. Proteases, which split one protein substrate into two polypeptide products, are an illustration of an enzyme that binds a single substrate and produces numerous products. Some connect two substrates, as DNA polymerase connecting a nucleotide to DNA.

Although these systems may include a number of intricate stages, the overall kinetics is usually determined by a single rate-determining step. This rate-determining process might entail an enzymatic reaction, a conformational change in the enzyme, or substrates, such those involved in the enzyme's release of product(s). Understanding the structure of the enzyme is beneficial for understanding kinetic data. For instance, the structure can provide clues as to the changes that take place throughout the reaction, how substrates or products bind during catalysis, and even the function of certain amino acid residues in the mechanism. When an enzyme's shape changes dramatically during its mechanism, it might be useful to compare the structure of the enzyme with and without bound substrate analogues that do not participate in the catalytic reaction.

Biological catalysts are not exclusively protein enzymes:

Many biological processes, including RNA splicing and translation, depend on RNA-based catalysts like ribozymes or ribosomes. The primary distinction between enzymes or ribozymes is that enzymes are made up of amino acids, whereas RNA catalysts are made up of nucleotides. Although ribozymes only carry out a smaller number of activities, their kinetics and processes may still be examined and categorised using the same techniques.

Action Energy and Reactions

Consider yourself trekking and having to climb a hill to get to the other side. You have to exert some effort to climb the slope. The effort required to climb a hill increases with its height. Similar to physical processes, molecules need energy to initiate a reaction in biological processes. For instance, in order to collide with other molecules and start a reaction, molecules need to have some kinetic energy, or velocity. No response will occur if collisions don't happen frequently or don't have enough kinetic energy.

The activation energy is the amount of energy needed to initiate a reaction. The speed of a reaction increases with decreasing activation energy. The steeper the slope, the quicker you climb the other side of the hill. In reactants and products, certain energies are present. To shift from reactants to products, the reactants would have to pass through a transition state, which frequently requires more energy. To reach this transitional stage, the system requires activation energy. The final step is for the products to minimise their energy and attain the completed product condition.

Catalytic Enzyme Kinetics

The study of chemical processes that enzymes catalyse is known as enzyme kinetics. While providing a different route from substrate to product with a lower Ea than other catalysts, enzymes do not change the equilibrium between the products and substrates.

Kinetics of Michaelis-Menten

The Michaelis-Menten model of enzyme kinetics describes how the concentration of an enzyme and its substrate affects the rate of an enzyme-catalyzed reaction. Consider a process where a substrate (S) and an enzyme (E) bind reversibly to create an enzyme-substrate complex (ES), which then undergoes an irreversible reaction to create a product (P) and release the enzyme once more [5].

$$S + E \rightleftharpoons ES \rightarrow P + E$$

In Michaelis-Menten kinetics, two crucial words are:

Vmax is the reaction's maximal rate when all of the enzyme's active sites are fully occupied by substrate. The concentration of the substrate at which the reaction rate is 50% of the Vmax is known as Km (sometimes referred to as the Michaelis constant). Km is a gauge of an enzyme's affinity for its substrate; the lower the value, the better the enzyme is at doing its task at low substrate concentrations.

The aforementioned reaction's Michaelis-Menten equation is:

$$v = \frac{V_{max}[S]}{K_M + [S]}$$

The initial substrate concentration has an impact on the initial rate of reaction (V), as shown by this equation ([S]). It presumes that the reaction is in a steady state, with a constant ES concentration.

Enzymes in Food Processing

A type of catalytically active protein is an enzyme. It has a better catalytic efficiency than inorganic catalysts. In addition to the typical properties of a chemical catalyst, enzymes provide the following benefits: high specificity, high catalytic efficiency, and low work condition. Enzyme engineering is a novel technique that combines chemical technology with enzymology theory. It is also a catalyst for the growth of the conventional chemical industry since it can overcome the inherent drawbacks of many chemical processes in a number of sectors.

In the past, plant extracts and animal offal were the main sources of the enzyme employed in food preparation. The majority of enzymes in use today come from microbial fermentation. The majority of enzymes used in food processing are somewhat refined, therefore their purity does not often need to be very high. The higher the purity, the better the impact, save in the particular applications, such proteolytic enzymes used in low-calorie beer. Glucoamylase is the most common enzyme used in food processing, followed by protease, esterase, lipase, oxidoreductase, and isomerase.

Amylases

Amylase is a digestive enzyme that is mostly released by the pancreas and salivary glands, while it is also very minutely present in other tissues. One of the earliest enzymes in human history to receive scientific study, amylase was initially described in the early 1800s. In the early 20th century, it was called amylase from the original name diastaste. The primary job of amylases is to hydrolyze the glycosidic linkages in molecules of starch, turning them to simple sugars. Amylase is an enzyme or particular protein that aids in the breakdown of carbohydrates. Your pancreas or salivary glands produce the majority of the amylase that is found in your body. It's normal to have a tiny quantity of amylase in your urine and your blood.

Proteases

The hydrolysis of peptide bonds is a typical chemical process that is efficiently carried out by proteases. There are several proteases that carry out somewhat different reactions from the majority of proteolytic enzymes, which break down -peptide bonds between naturally existing amino acids.

Phenolase

An enzyme known as an oxidoreductase that catalyses the oxidation of phenols and other compounds that are related. Numerous plants, fungi, and microbes have this enzyme. The

oxidation of several compounds, including tyrosine, is catalysed by it. Fruit browning caused by this enzyme is visible when fruits like apples, bananas, and potatoes are chopped or damaged.

Glucose Oxidases

Glucose oxidase is used in numerous commercial processes, including those that enhance food components' colour, flavour, and persistence, remove glucose from dried eggs, and remove oxygen from various juices and drinks. Gluconic acid and hydrogen peroxide are produced when the enzyme glucose oxidase interacts with glucose, water, and oxygen. The quantity of oxygen used while the hydrogen peroxide is oxidising a chromogen or measuring the amount of glucose present may then be determined.

Lipoxygenases

The generation of corresponding hydroperoxides from polyunsaturated fatty acids like linoleic acid and arachidonic acid is catalysed by lipoxygenases (LOXs), which are dioxygenases. A class of nonheme iron-containing enzymes known as lipoxygenases (LOX) catalyses the deoxygenation of PUFAs to produce hydroperoxyl derivatives, such as hydroperoxy-eicosatetraenoic acids.

Oxidases of Xanthine

An essential enzyme known as xanthine oxidase (XO) is responsible for catalysing the conversion of uric acid, which is excreted by the kidneys, from xanthine and hypoxanthine, respectively. Hyperuricemia is brought on by excessive production and/or insufficient excretion of uric acid. The techniques of isolation, the measurement of xanthine oxidase activity, as well as the impact of plant extracts and their components on it are all covered in length in this work.

CHAPTER 14

BASICS OF PEROXIDASE

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A class of enzymes known as peroxidases is responsible for catalysing the oxidation of a substrate by either hydrogen peroxide or an organic peroxide. Peroxidases are enzymes that can convert other hydroperoxides, including hydrogen peroxide, into water. This technique involves oxidising a number of substrates. According to the chemistry of the active site interacting with peroxide, peroxidases are classified as heme and nonheme peroxidases. The active site of the former has a heme catalytic core, whereas the latter has a selenol or reactive thiol. Here, certain peroxidase enzymatic characteristics and biochemical characteristics are discussed.Without eating digestive enzymes, people cannot adequately digest food, especially when the meal has already been prepared. Because the food's enzymes were damaged during preparation and boiling. Unless they are grown organically, even fresh fruits and vegetables are frequently irradiated, which kills their enzymes.

Currently, enzymes are utilized in a variety of food items and manufacturing processes, and new areas of application are constantly being developed. Evidence demonstrates that persistent and committed research efforts are being undertaken to increase the effectiveness and variety of this use of biological agents. Enzyme immobilization has been a crucial strategy for preparing proteins for use in food applications while also enabling the enhancement of their catalytic activities. Enzyme technology presents a significant opportunity for many food sectors to assist them overcome the issues they will face in the future in a world with a population that is growing quickly and that is close to running out of many natural resources.

Enzyme Immobilization

In the past, enzymes in soluble or free form would interact with substrates to produce products. Since enzymes are unstable and cannot be retrieved for reuse, such usage of them is wasteful, especially for industrial applications. The process of enclosing/anchoring the enzymes and cells in or on an inert support for their stability or functional reuse is known as immobilization of enzymes or cells. This method increases the effectiveness and affordability of enzymes for industrial usage. In enzyme technology, some employees see immobilization as the goose that lays the golden egg. Enzymes that have been immobilized maintain the structural shape required for catalysis.

Immobilization Strategies

Despite the fact that using enzymes has several benefits over using conventional catalysts, there are a few real issues with using them in industrial settings. Because enzymes are often costly, the cost of isolating and purifying them is significantly higher than the cost of creating regular

catalysts. They have a protein-like structure, which makes them extremely vulnerable to different denaturing circumstances when separated from their normal surroundings. They may act as inhibitors that raise their costs due to their sensitivity to process variables including pH, temperature, or chemicals at trace quantities.However, unlike traditional heterogeneous chemical catalysts, most enzymes work in homogeneous catalysis systems while dispersed in water, which results in product contamination and prevents their recovery in the active state from the majority of reaction mixtures for reuse.

The employment of an immobilization tactic is one of the most effective strategies suggested to get around these restrictions. Enzymes are fastened to or contained by solid supports by the technological process of immobilization, resulting in a heterogeneous immobilized enzyme system. Enzymes that have been immobilized imitate the way that they normally function in living cells, where the majority of them are anchored to organelle, membrane, and cytoskeleton components. The solid support systems often preserve the enzymes' activity by stabilizing their structure. Immobilized enzymes are therefore more durable and resistant to environmental changes than unbound enzymes in solution.Additionally, heterogeneous immobilized enzyme systems provide simple enzyme and product recovery, repeated enzyme reuse, continuous enzymatic process operation, quick reaction termination, and a wider range of bioreactor design options. However, due to a significant difficulty in reaching the substrate, most often immobilized enzymes exhibit decreased activity and typically have larger apparent Michaelis constants when compared to free enzymes.

The Main Techniques for Immobilizing Enzyme

A crucial step in the immobilisation process is selecting the best immobilisation technique. It is crucial in identifying the characteristics and activity of the enzyme in a certain process. Chemical and physical immobilisation techniques may be split into two broad types. Weak monovalent interactions, such as van der Waals forces, hydrophobic interactions, hydrogen bonds, affinity binding, ionic binding of the enzyme with the supporting material, or mechanical containment of the enzyme within the support, are what distinguish physical methods from other types of interactions. When using chemical procedures, the supporting material and the enzyme establish covalent connections through the use of ether, thio-ether, amide, or carbamate bonds. For the immobilisation of enzymes, there are five main techniques: adsorption, entrapment, encapsulation, covalent binding, and cross-linking.

Method of Immobilization

To prevent deactivation of the enzyme's surface-located active site, it is crucial to select the proper immobilisation technique. Knowing each enzyme's active sites will enable you to select the best strategy to stop a reaction with it.Additionally, through immobilisation technology, these active sites can be protected; in certain situations, this is accomplished utilising an enzyme-substrate or a particular inhibitor. Stability, enzyme catalytic activity, and economic considerations are only a few of the numerous aspects that go into choosing an appropriate immobilisation technique.Adsorption One of the simplest immobilisation techniques is physical adsorption; in this technique, the enzyme is adsorbed on the surface of the matrix, which may be

an organic or inorganic matrix. This adsorption technique relies on weak connections between the enzyme and solid matrix, such as van der Waal's force, electrostatic and hydrophobic contacts, hydrogen bonds, or ionic bonds.

The most used technique for immobilising enzymes is covalent binding. For this approach to work, functional groups like amino, carboxylic, hydroxy, and sulfhydryl groups that are located on the surface of the support material must create a stable covalent link with the functional groups in the enzyme.Sometimes the support components must be activated before the covalent link with the enzyme can be formed. The primary drawback of this approach is the possibility that an enzyme's active site may be used to carry out the reaction (amino group). This denotes the inactivation of the enzyme. Therefore, it is essential to select the appropriate immobilization technique for each unique response.

One of the simplest ways to immobilize an enzyme is through entrapment. The enzyme is physically constrained inside the network of support material using this technique. Since there is no chemical contact between the enzyme and the support material, enzyme entrapment can increase the stability of the enzyme without reducing its activity. The choice of support material and its pore size, which have an impact on how restricted enzyme and substrate react, are the crucial parameters in this approach. The enzyme is free to move in solution but is constrained inside the support material, which is how the entrapment approach differs from others like adsorption and covalent binding.

Application of Immobilized Enzymes and Cells

In many industrial and medicinal processes, as well as in the manufacture of food and the investigation of concepts in microbiology, biochemistry, and other related fields, enzymes are the important participants. The enhancement of enzyme activity, repeatability, efficiency, and stability throughout industrial operations is the subject of heated debate. Through the use of immobilisation technology, enantio- and regioselective molecules have been created for medicinal uses.

Applications in Industry

Researchers have made several attempts to lower product costs by recycling the enzymes in industrial settings. They discover that this is feasible by reusing enzymes while preserving their stability and efficiency using an immobilisation method. People noticed that the number of studies on the application of immobilisation technology in industry between 1997 and 2018 that were published in the Science Direct database is continually growing, highlighting the significance of this technology in the industry.

Application in the Food Sector

The food sector may make use of the purified enzymes. During the purifying procedure, it could get denatured. However, the immobilised enzymes are stable and simple to separate. Enzymes that have been immobilised can be utilised to make syrups. In order to create glucose from waste products, immobilised -galactosidase has been used to break down the lactose in whey. It may also be used to create lactose-free milk. Additionally, it may be used to make baker's yeast. The

polymeric matrix may be used to encapsulate essential oils that have nutritional and health advantages, such fennel and cumin, and employ them as preservatives. Essential oils of fennel and cumin can be utilised in foods including bread, drinks, pastries, pickles, and cheese.

Dairy Industry

The dairy industry's use of cell immobilisation technology has received much study. It alters the physiology of the cell as well as the effects of cell immobilisation technology on the metabolism of lactose or citrate. This method may be used to make starter for the dairy industry, includes acidifying raw milk before ultrafiltration, making yoghurt, fermenting cream, and making cheese. Additionally, dairy foods may conjugate linoleic acid via lipase. The problem of lactose intolerance, which is present in many nations globally, has stimulated interest in the creation of dairy methods to hydrolyze lactose contained in dairy products. Using the enzyme -galactosidase immobilised on a polymeric substance, lactose may be transformed to glucose and galactose in this process. Compared to lactose, these monosaccharide sugars are sweeter, soluble, and digestive.

Textile Sector

It is a long-standing industry that makes up a sizable chunk of the economies of many nations. In this business, microbial enzymes are of tremendous interest. These enzymes, including amylase, laccase,.cellulase,.cutinase, or pectinase, are utilised in a variety of textile applications, including scouring, bio-polishing, finishing denim, as well as wool processing design. Cellulase enzymes have been the most widely employed enzyme in this business from the older era to the present. The free enzymes are unsuitable in such harsh settings since this business demands high pH and temperature levels. As a result, immobilised enzymes can respond to challenging circumstances and preserve their stability throughout several cycles. Cellulase enzyme is used to create synthetic polymethyl methacrylate nanoparticles that are covalently linked.

Treatment of Waste Water

One of the most serious environmental issues that warrants concern globally is heavy metal contamination. Heavy metals including Cr, Cd, Zn, Pb, Ni, Ag, as, and Hg may be present in industrial effluent. Precipitation, solvent extraction, ion-exchange separation, and solid-phase extraction are the most involved methods used to remove matrix interference. To clean up wastewater that had been contaminated with heavy metals, biosorbents such immobilised algae were utilised. These biosorbents are described as the selective separation of species that are metal-soluble and cause bacterial organisms like cyanobacteria to immobilise metals.The metal ions are instead removed from the water by immobilising them on the surface of activated bio-polymeric materials, such as carrageenan and alginate, using a technique known as immobilisation technology.

Use of Pharmaceuticals

Enzyme production or processing for medication usage is a crucial secondary component of the pharmaceutical business today. In practically every scientific centre of pharmacy in the world, efforts were made to use the advantages of enzymes as medicines. In light of the number of

studies dealing with immobilisation technology in pharmaceutical applications that were published in the Science Direct database between 1997 or 2018, we discovered that it is continuously increasing, demonstrating the significance of this technology in the pharmaceutical industry. Since 1990, enzyme immobilisation technique has been applied to medical procedures. Artificial organs and immobilised enzymes can both be employed in the medical sector to identify or cure diseases.

To increase biocompatibility, cells can also be included into coatings for artificial materials. Through electroporation, enzymes can be encapsulated and employed in the medical industry. This technique is the simplest available for immobilising an enzyme in this sector, and it is reversible, allowing the enzyme to be recovered.

In Clinical Medicine, Immobilized Enzymes

Due to the fact that they carry out intricate chemical processes under physiological settings, enzymes can be employed as therapeutic agents. Numerous of these interactions take place in organisms that are healthy and normal but are vulnerable in the presence of sickness or patients who lack moral character. A biological function that is not inherited by the organism can be conferred on it by an externally administered enzyme. Consequently, the immobilised enzyme has various benefits over the soluble one. With regard to their clinical use, several enzymes have been studied. For instance, bilirubin oxidase that has been immobilised was utilised to eliminate bilirubin from the circulatory system.

Diabetics

It has been reported that glutaraldehyde is used to activate the immobilised glucose oxidase on a polycarbonate membrane that has been modified via urethane coupling with poly (l-lysine). The immobilised enzyme has greater pH stability and heat stability than the non-immobilized enzyme. The quantity of glucose oxidase adsorbed on the typical polycarbonate membrane was quite little, as seen by a comparison between both the enzyme activity and the immobilisation technique. In contrast, there was no leakage seen when covalent interaction with aldehyde groups in the generated membrane string.

Bioreactors and Fermentation

An example of a fermentation vessel is a bioreactor, which is employed in the synthesis of numerous chemicals and biological processes. In order to remove the waste biomass of cultivated microorganisms as well as their products, it is a closed container with suitable arrangements for aeration, temperature control, agitation, and pH control.

The following should be included in a bioreactor:

1. Agitation (for mixing of cells and medium).
2. Regulation of variables like aeration, pH, pressure, temperature, nutrient feeding, or liquid levelling.
3. Aeration (aerobic fermenters), for O_2 supply.
4. Withdrawal of cells/medium, sterilization, or maintenance of sterility.

Design of Bioreactors

A bioreactor's design and method of operation are based on the creation of an organism, the ideal circumstances needed for the formation of the intended product, the value of the product, as well as its scale of production.The productivity of a bioreactor may be increased, and it can produce goods of greater quality for less money. A bioreactor is a device that includes a number of features, including an agitator system, a foam control system, an oxygen delivery system, and a number of other systems, including sampling ports, cleaning and sterilization systems, lines for charging and emptying the reactor, and systems for controlling temperature and pH, as shown in Figure 14.1.

The following crucial characteristics must exist in the substance used to make a bioreactor:

1. It shouldn't be acidic.
2. Toxic compounds shouldn't be added to the fermentation medium.
3. It must be able to withstand the steam sterilizing procedure.
4. It need to be capable of withstanding high pressure and enduring pH variations.

Principle of a Bioreactor

1. In order for microorganisms to develop optimally and create metabolites for the biotransformation or bioconversion of substrates into desired products, the bioreactor is essential to any biochemical process.
2. Reactors can be designed or created based on the needs of the organisms employed for growth.
3. Reactors are devices that may be used to turn materials with biological origins into palatable goods.
4. They may be utilized to produce other enzymes and carry out other bio-catalysis procedures.

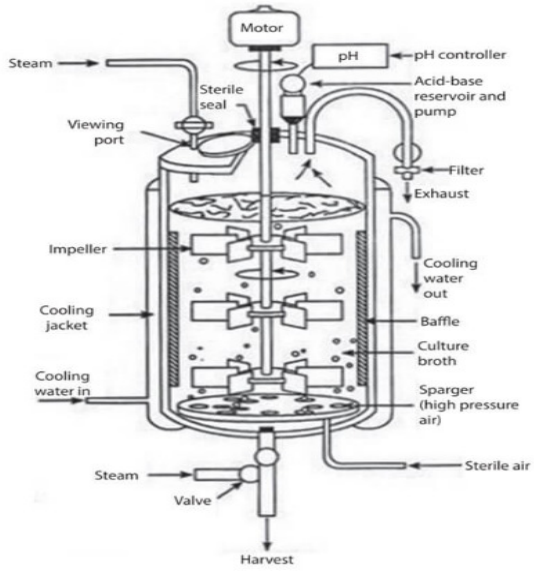

Figure 14.1: Illustrate the Pictorial Representation of Bioreactor.

Fermentation is an anaerobic chemical process that breaks down molecules like glucose. More specifically, fermentation is the foaming that happens during the creation of wine and beer, a procedure that has been around for at least 10,000 years. Though this wasn't understood until the 17th century, the foaming is caused by the development of carbon dioxide gas. Louis Pasteur, a French scientist and microbiologist, understood that ethyl ethanol and carbon dioxide are not the sole results of fermentation and used the phrase in a restricted meaning to describe the changes caused by yeasts and other microbes growing anaerobically (without air).

Anaerobic Molecular Breakdown

It was established in the 1920s that muscle extracts accelerate the synthesis of lactate from glucose in the lack of air and also that muscle produces the same intermediate chemicals generated by grain fermentation. Thus, a significant generalization emerged: fermentation events happen in many different cases of glucose use and are not exclusive to yeast activity.

Industrial Fermentation

Beginning with appropriate microorganisms and predetermined circumstances, such as carefully adjusting nutrient content, industrial fermentation processes are possible. Numerous products are produced, including glycerol,.alcohol, and carbon dioxide when different sugars are fermented by yeast;.acetone, monosodium glutamate,.butyl alcohol, lactic acid,.and acetic acid when various bacteria are involved; and citric acid, gluconic acid, and trace amounts of vitamin B12, antibiotics, and riboflavin (vitamin B2) when mould is involved. The fermentation of starch or sugar results in the production of ethanol, which is a significant source of liquid biofuel.

A material collects moisture from the air through the process of deliquescence, which continues until the substance dissolves in the water and produces a solution. Deliquescence happens when the solution's produced vapour pressure is lower than the partial pressure of airborne water vapour. If the air is sufficiently humid, all soluble salts will dissolve. A material is referred to as hygroscopic if it can collect moisture from the air without necessarily dissolving. The deliquescence of calcined calcium chloride contributes to its efficiency in settling road dust. It absorbs more water than it can hold when dispersed as flakes or powder, creating a liquid that keeps the road moist.

CHAPTER 15

ENZYME REACTORS

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The use of enzymes as process catalysts accounts for more than 80% of their economic worth. The conventional method of employing enzymes has been hydrolytic reactions, which are mostly carried out with the enzyme dissolved in an aqueous solution. This technique still accounts for a sizable portion of enzyme operations. The utilisation of enzymes in organic synthesis has, however, expanded to previously unheard-of proportions in recent decades.Enzyme reactors can run continuously or in batches, and fed-batch operation has also been proposed.Despite their widespread use, batch processes involving the enzymes (typically hydrolases) dissolved in an aqueous reaction medium have a number of drawbacks because the enzymes are poorly stable and difficult to recover in such systems, resulting in low productivity; in addition, such processes are characterised by a relatively low added value, so process optimization is crucial for remaining competitive. Enzyme stabilisation during reactor operation is a big problem since poor stability is typically the limiting factor in any enzyme process. Among the several ways for enzyme stabilisation, enzyme immobilisation is the most pertinent. Immobilized enzymes can be utilised in batch operations, although in this instance, the enzyme is recovered for usage in consecutive batches until the accumulated inactivation necessitates the replacement of the used biocatalyst. The specific productivity (mass of product/mass of biocatalyst duration of operation) increases as a result, and the bioreactor design is made more adaptable to the unique requirements of a given process.

Reactors for Immobilized Enzymes

However, the other physical properties, such as form, shape, porosity, pore size distribution, swelling capability, and charges, are also very important because they affect the kinetic process. Indeed, the reaction rates of the immobilized enzyme depend on the enzymatic intrinsic activity, the substrate accessibility to the active sites, the amount of the loaded enzyme, substrate concentration and diffusivity.

Supports for Immobilization

The characteristics of the support materials are directly connected to the primary issue in the enzyme immobilisation procedure. The optimal support material should be known for its chemical group, three-dimensional porosity structure, and high biocompatibility. The mechanical strength may be altered by all these characteristics, which also make the material stable over a wide range of temperature, pH, ionic strength, and organic solvents.

Even while not all enzymes and their associated applications have universal acceptance, most materials that are thought to be suitable for immobilised enzymes have several desired qualities.

All immobilised enzyme supports have been examined, and they are often divided into organic and inorganic natural and manmade materials. Transmission electron microscopy, scanning electron microscopy, and Fourier transform infrared spectroscopy are used to analyse the shape and characteristics of supports. By using techniques like X-ray diffraction, elemental analysis, thermogravimetric analysis, and others, the component properties of supports are identified.

Types of Reactors

1. Batch Reactor
2. Plug Flow Reactor (P.F.R)
3. Continuous Stirred Tank Reactor (C.S.T.R)
4. Semi-Batch Reactor.

A batch reactor is a form of non-continuous reactor that consists of a closed vessel in which reactions take place. Initially, the reactants are supplied into the reactor all at once. The vessel has an agitator in it. The agitator's job is to fully combine the reactants such that their contact causes them to react effectively and yield products.Cooling coils are frequently used in batch reactors to handle exothermic processes.

The batch reactor incorporates features for heating the reaction mixture so that it may be used with endothermic processes.A transient, non-steady reactor is the batch reactor. It implies that time affects how much conversion occurs inside the reactor. The batch reactor's nature is quite consistent thanks to the agitator. It implies that position within the reactor has no bearing on the degree of conversion. Any spot in the reactor's volume will experience an equal amount of reaction at any given time

Advantage

The adaptability of using a batch reactor is its main benefit. Chemical reactions between a wide ranges of reactants can occur in the same batch reactor. Batch reactors are particularly useful when the reaction yields a large number of products. In laboratories, batch reactors are frequently employed to investigate the kinetics of liquid phase reaction systems.

Continuous Stirred Tank Reactor (C.S.T.R)

Another name for a continuous stirred tank reactor (C.S.T.R.) is a mixed flow reactor (M.F.R). The reaction also takes place in a closed tank in this reactor. Additionally, the tank features an agitator to completely mix the reactants. It differs from a batch reactor in that its name already makes clear that it is a continuous piece of machinery. The reactants enter the reactor at a specific mass flow rate, react for a period of time determined by the reactor's space time, and then produce products.

At the same mass flow rate, the products exit the reactor. To process one reactor volume, it takes one space time. The C.S.T.R is reliable machinery. It implies that the amount of conversion is independent of time. The agitator evens out the concentration inside the reactor. It implies that the degree of conversion is independent of location. The reactor's volume determines how much conversion will occur.

Flow Plug Reactor (P.F.R)

A continuous tubular reactor (CTR) is another name for a plug flow reactor (P.F.R.) (C.T.R). In an idealised model, the reaction mixture's profile may be thought of as being composed of a number of plugs, each of which has a uniform concentration. It is assumed that there is no axial mixing in the idealised P.F.R model. It denotes the absence of back mixing within the reactor.

Semi-flow reactors are semi-batch reactors. It is a batch reactor modification. Additionally, it is a closed vessel with an agitator to fully mix the reactants. The distinction is that whereas one of the reactants is initially fully charged in the reactor, the other reactant is constantly charged as time goes on.

Advantage

When performing several reactions, employing a semi-batch reactor gives us better control over the yield or selectivity of the end products. When performing an exothermic reaction, this reactor is very helpful since the continuous flow of the other reactant may be changed to more effectively regulate the exothermic process.

Advances in Reactors

In controlled environments, bioreactors provide nutrients and biomimetic stimuli to influence cell growth, differentiation, or tissue formation. They have been frequently used to promote the development of red blood cells, mesenchymal stem cells, and induced pluripotent stem cells. By providing precisely controlled conditions to control cell behaviours, the ability to control the spatiotemporal delivery of the biological, biochemical, and biophysical signals that control tissue growth confers a number of advantages for engineering 3D tissues in comparison to standard cell culture techniques. Bio production processes have long been automated, and in the near future, automation will likely become much more prevalent.In order to transform this raw data into information that can be utilised to regulate and enhance the process and product, data integration is required to handle the enormous rise in product data that results from this expansion.

Numerous industries, including healthcare, space exploration, industrial biotechnology, or environmental protection have utilised bioreactors in various ways. Strong market demand encouraged the creation of innovative bioreactor geometries, process control schemes, and the physical evolution of the control system. A hierarchical structure control system (HSCS) for bioreactors has emerged as the dominant physical structure with high efficiency and resilience following the introduction of digital computers to bioreactor process control. However, the HSCS for bioreactors has limitations that make a more comprehensive control system solution necessary.The creation of a flat organisational control system (FOCS) for bioreactors based on parallel distributed smart sensors and actuators may offer a more succinct solution for process control in bioreactors given the rapid advancements in sensors, equipment, and information technology. In order to further enhance the effectiveness, reliability, and economics of bioprocess control, we will review the development of the physical structure of bioreactor control systems and go over the characteristics of a novel FOCS for bioreactors as well as connected smart sensors and actuators and their application scenarios.

Automation and Design of the Bioreactors

Simply said, a bioreactor is a container where basic materials are transformed into new products under carefully regulated working conditions. Bioreactors come in three main flavours: batch, continuous, and semi-batch. Three batch bioreactors were planned, built, and automated for this project. Fruit juice served as the reactors' feedstock, enabling the fermentation of glucose into ethanol to take place without the need for further substrate preparation. As part of their senior capstone project, engineering technology students built and automated the functioning of the bioreactors. The three reactors were finished by three distinct teams of engineering technology students over the course of three academic semesters.The environmental technology students collaborated extensively with biology students in environmental microbiology during one of the later semesters. Together, the students from the two disciplines were in charge of designing the operational parameters, determining the product's ethanol percentage, and monitoring the feedstock. The first bioreactor (Bioreactor 1) did not have any components that were prefabricated.

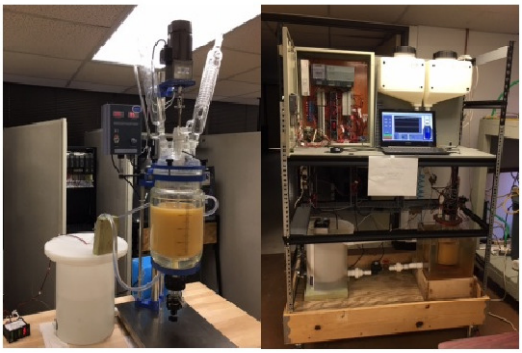

Figure 15.1: illustrate the Pictorial Representation of Bioreactor.

All of the parts were recognised by the students, who then combined them into a full and functional system. Figure 15.1 depicts a bioreactor of this type. The vessel for the other two bioreactors was already built. The reactors also had rudimentary temperature control devices and a motor for mixing the reactor's contents.

Smart Sensor and Actuator Applications in New Bioreactor Control Systems

The aforementioned bioreactor control systems for which sensors have been crucial are bioreactors. There are still some process metrics, particularly those relating to the biochemical and physiological features of cultures that are difficult to quantify, even though the majority of physical and chemical process parameters may be directly seen using particular functional sensors.As has been addressed in several outstanding reviews, numerous unique sensors and state-of-the-art PATs have been developed as the foundation of smart sensors. Studies on optical sensors or spectrographic analytical methods have garnered a lot of interest among them. Numerous sensors and PATs have been designed to monitor these process parameters since cell morphology is a crucial physiological trait of cultures that may have a significant influence on the end products

The use of flow cytometry-based technologies for the monitoring of various apoptotic phases of mammalian cell cultures throughout a production process was described in one research. The morphology and vitality of Penicillium chrysogenum filamentous fungal cultures as well as the population heterogeneity in response to shifts in substrate availability in Escherichia coli and *Saccharomyces cerevisiae* chemostats have all been studied using flow cytometry-based techniques.Another useful method for examining population heterogeneity is in situ microscopy. In situ microscopy was used in one study to track the budding index in real time in batch cultivations of *S. cerevisiae*. In many bioprocesses, population heterogeneity resulting from inhomogeneous culture conditions is a frequent occurrence, and this heterogeneity includes changes in the intracellular concentrations of significant metabolic intermediates in addition to cell shape. In order to design bioprocesses and produce them on a big scale, it is important to monitor vital metabolites like lipids, proteins, or starch inside the cells. Created a Nile red test that was automated and allowed for the high-throughput measurement of microalgal lipid synthesis for the development of bioprocesses. Several organizations also reported using non-invasive Raman spectroscopy to monitor the generation of mAb and lipids throughout the development of bioprocesses. For feedback control, non-invasive Raman spectroscopy has also been used to monitor significant parameters like glucose content in a bioreactor.

Fermentation Technology

The word "fermentation" refers to the growth of microbial cells and the production of products in microaerobic, aerobic, or anaerobic environments. Microaerobic refers to air that is initially present but is then used up or displaced as microbial growth occurs. Anaerobic refers to a condition where oxygen is removed and intentionally excluded from the fermentation media because it is toxic to the cells. Aerobic denotes a condition where air is intentionally mixed with the medium. As a result of the oxidation of monosaccharides, notably glucose under both aerobic and anaerobic circumstances, metabolites are released from the inside of microbial cells into the surrounding media and accumulate in the medium.

The entire fermentation process may be broken down into three steps.

1. Stage I: Upstream processing, which includes liquid medium preparation, removing particulates and inhibiting compounds from the medium, sterilisation, air purification, etc.
2. The second stage is fermentation, which includes using biological agents like bacteria to transform materials into the desired product.
3. Stage III: Downstream processing, which includes removing the cells from the fermentation broth, concentrating the desired product, disposing of or recycling the trash, and purifying the cells.

A fermenter is necessary for the fermentation process because it gives the organism living inside of it the ideal pH, oxygen, temperature, and other environmental conditions for growth. A fermenter is a container for the development of microorganisms that allows for the supply of the conditions essential for the maximum production of the desired products while preventing contamination.

Different Fermentation Methods

Submerged Agriculture

The effective manufacture of high-quality end products and the achievement of optimum productivity and output are ensured by submerged culturing of microbial cells in bioreactors. Different kinds of microorganisms are cultured in industrial bioreactors that may be run in batch, fed-batch, or continuous mode, yielding a wide range of products. The following discussion covers several strategies for submerged microbe culture in bioreactors.

Batch cultivation

The volume of the culture broth in the bioreactor is theoretically constant throughout cultivation in batch culture, which represents a closed system in which the medium, nutrients, and inoculum are added to the bioreactor at the beginning of cultivation, typically under aseptic conditions. In practise, however, small deviations in culture volume are brought on by a low feed rate of acid/base solutions to maintain a desired pH level and by sampling or introducing air/gas into the culture; on ballast. A known quantity of viable cells are typically introduced into the bioreactor, which is already filled with sterile medium including all nutrients, before the start of batch production. The cell culture follows the specified classical growth curve after inoculation, which is broken down into four basic stages.

Batch culture systems provide the following benefits:

1. Because the growing phase is brief, there is little chance of contamination or cell mutation.
2. Reduced capital expenditure for the same bioreactor volume compared to continuous operations.
3. More adaptability with various biological/product systems.
4. Higher levels of raw material conversion as a result of a time of regulated growth
5. Processes that create many products utilizing a single reactor.

Fed-Batch Cultivation

Fed-batch culture is a semi-open method in which the product is held inside the bioreactor while one or more nutrients are introduced aseptically and gradually. During this period, the amount of the culture broth in the bioreactor grows. The main benefits of fed-batch cultures over batch cultures are (a) the ability to extend the time it takes for a product to be produced, (b) the ability to achieve higher cell densities and thereby increase the amount of the product, which is typically proportional to the concentration of the biomass, (c) the ability to increase yield or productivity by carefully timing the addition of nutrients, and (d) the ability to extend productive cultivation over "unprofitable periods" when the bioreactor is not in use.

Continuous Cultivation

The concept of continuous culture is an open system in which nutrients are continuously and aseptically added to the bioreactor while the culture broth, which contains cells and metabolites, is removed at the same time. The volume of the culture broth is constant because of a constant

feed-in and feed-out rate.A chemostat is employed in continuous culture, which is characterised by a constant specific growth rate of cells that is equal to the dilution rate and is regulated by the availability of the limiting nutrient. The rate of development will thus be 90% of the maximum if 100 grammes per litre of the limiting nutrients are needed for maximal growth but only 90 grammes per litre are provided, even when alternative continuous operating methods like turbidistat a constant concentration of biomass controlled by the dilution rate.

Fermentation of Solid Substrates

In systems where microorganisms are cultivated on the surface of a concentrated water-insoluble substrate with a low amount of free water, these processes are referred to as “solid substrate fermentation” (SSF) or “solid substrate cultivation” (SSC).

Using various substrates and microbes, this method was established in the Eastern nations, where it has been used for generations to produce traditional delicacies like soy sauce, miso, and sake. It has not been extensively utilised in Western nations, and its principal applications are for the production of industrial enzymes, certain food items, or feed additives.

Types of Sterilization

1. Physical method
2. Chemical Method
3. Biological Methods

Sterilization is the removal of living germs from materials. It is done to sterilise objects and preserve them for a long period.

When something is not sterilised, it might infect the person using it. As a result, it shouldn't be assumed. There are several sterilising techniques, such as:

Heat Sterilization

The heat employed in this method of sterilising eliminates all microorganisms, making it the most popular. The length of heating and heat temperature have an impact on the degree of sterilisation. The length of heating increases as temperature does. The heat sterilisation method may be further classified into two categories:

Moist Heat Methods

Boiling is used in this instance to apply heat, along with other techniques including pasteurisation and the use of steam. Metal objects including surgical scissors, custom trays, and needles are boiled.

To eradicate any microorganisms, the materials are cooked. On the other hand, pasteurisation is a process that involves heating milk three or four times to 60 or 72 degrees. The items that need to be sterilised are exposed to steam in autoclave steam heating apparatus. Temperatures of up to 115 degrees are used for the procedure for an hour. Due of its ability to eradicate bacterial spores, which are inactive bacterial forms, it is the most used approach for sterilising pharmaceuticals

Dry Heat Methods

Flaming, cremation, hot air ovens, or radiation sterilisation are used to treat materials. Flaming is the practise of placing metallic objects over a flame over a period of time, such as scalpels or needles. All bacteria will be killed instantly by the flame. Particularly for inoculating the loops used in microbe cultures, incineration is employed. All bacteria are destroyed when the metallic end of the loop is burned red hot on a flame. Packets of materials are exposed to radiation using the radiation technique. Ionizing and non-ionizing radiation sterilisation are the two forms of radiation. While the latter needs the operator to wear protective gear, the former is safe for the individual doing the treatment. Dry materials like glass and powder work best with the hot air approach. They are heated in a hot air oven on racks until disinfected.

Sterilization with Chemicals

With this technique, poisonous fumes are used to sterilise the goods. Use bacterial filters for sterilising heat-sensitive liquids. This kind of sterilisation employs one of three filter types:

Seitz filters are pad-like and thicker than membrane filters; they are constructed from materials like asbestos. Seitz filters don't break while they're filtering. The filter, however, may wind up absorbing the solution. Sintered glass filters, which are formed of glass and cannot absorb liquids, are an alternative to Seitz filters.

They are brittle and breakable, though.Membrane filters: composed of cellulose, these tiny filters can be utilised for online sterilisation during injections. In between the needle and syringe is the membrane. However, this kind of filter is prone to rupture, which results in inadequate sterilisation.

Strain Development of Important Industrial Microorganisms

A combination of heat, chemicals, irradiation, high pressure, and filtration can be used to sterilise objects, such as by the use of steam under pressure, dry heat, UV radiation, gas vapour sterilants, chlorine dioxide gas, etc. Working in a lab requires effective sterilising techniques, and failure to do so might have serious repercussions, including the unexpected loss of life.

1. *Using moist heat to sterilize:* When using steam sterilisation, moderate pressure is used. As a way to achieve a higher temperature, steam is used under pressure. To avoid issues like steam overheating, inadequate steam penetration into porous loads, inappropriate air removal, etc., it is imperative to ensure that the right kind of steam is being used.
2. *Selection of high-yielding mutants:* In this method, a population of microorganisms is searched for variations that outperform the original strain in terms of product production. By plating cultures on media containing a selection agent that is poisonous to the original strain but not to the mutant, high-yielding mutants are most frequently selected. Colonies from the plates can then be grown in liquid culture, and mutants with high yield can subsequently be identified by selecting for growth on minimum medium.
3. *Genetic engineering:* This strategy entails changing a microorganism's genetic makeup to increase its output. Inserting genes from other species that produce enzymes that catalyze the creation of the desired product is one such tactic.

4. *Metabolic engineering:* This approach involves altering the metabolic pathways of a microorganism to redirect its resources towards the production of the desired product. One common strategy is to knock out genes that are not essential for product formation and replace them with genes from other organisms that encode enzymes that catalyze the production of the desired product.

Enhancing a certain cannabis strain's qualities is known as "strain improvement." Genetic alteration or selective breeding can be used to accomplish this. To create a plant with the desired characteristics, such as a high THC content, big buds, and a quick blooming time, is the aim of strain improvement. Breeders employ a range of techniques, including as backcrossing, crossing, or hybridization, to enhance strains.
