ENZYMOLOGY

Industrial and clinical applications excluding Diagnostic clinical enzymology

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CONTENTS

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Introduction

Since the beginning of human history, man has used enzymes indirectly. The fermentation of sugar to ethanol in the preparation of beer and wine, production of vinegar by the oxidation of ethanol, curdling of milk by lactose fermentation are thousands of years old processes where catalytic activities of enzymes are responsible for chemical transformations. Probably the first application of cell free enzyme was in cheese making where rennin obtained from calf stomach was used. The protease rennin which coagulates milk protein, has been used for hundred of years in cheese preparation. The first commercial enzyme was probably reported in Germany in 1914. Use of trypsin, the protease isolated from animals, was shown to improve washing power of detergent over traditional products. Success in the formulation of improved quality of detergent triggered efforts towards the selection of proteases suitable for application in detergents.

Subsequently, a breakthrough in the commercial use of enzyme occurred with the introduction of microbial protease in washing powder at an affordable cost. The first commercial alkaline protease from *Bacillus* sp. was marketed in 1959 and production of enzyme added detergent soon became a big business within a few years. During the period when use of alkaline protease in detergent became popular, use of enzymes in food processing industries also gained momentum in parallel. Fruit clarifying enzyme, called pectinase, was used in fruit juice manufacturing units since 1930. Enzymes hydrolyzing starch into dextrin and glucose largely entered the food industry in 1960 and more or less completely replaced acid process of starch hydrolysis. Starch hydrolyzing enzymes (α-amylase and amyloglucosidase) for the production of glucose soon became the second largest used group of enzymes in industry after detergent protease.

Enzymes may be extracted from any living organism. Sources of commercial enzymes cover a wide range, from microorganisms to plants to animal sources. But for various reasons, microorganisms became the major source of enzymes. In commercial enzyme production, fungi and yeast contribute about 50%, bacteria 25%, animal 8% and plant 4% of the total. Microbes are preferred to plants and animals as they are cheap sources, their enzyme contents are predictable and growth substrates are obtained as standard raw materials. In addition, genetic engineering has opened a new era of advanced enzyme technology. Recombinant DNA technology has made it possible to obtain enzymes present in valued sources, to be synthesized in easy growing microorganisms and also to produce tailor-made enzyme proteins with desired properties as per customers' requirements. Enzymes retaining activity under extreme conditions of temperature, pH and salt concentrations, partially active in organic solvents are all becoming a reality. The prospects of the enzyme industry look very bright with increased market position for existing use, new use of known enzymes and new enzymes having novel industrial applications.

According to a recent release by Business Communication Company Inc. Study RC-147 NA, Norwalk, CT 06855, USA on "Industrial Enzyme Products, Technologies and Applications", the world wide total industrial production of enzymes was of valued at 1.5 billion US dollars in 1997 and it gained an average annual growth rate of 4.0%. Food and animal feed applications of enzymes are constantly dominating the use of industrial enzymes on a worldwide basis.

Large volumes of industrial enzymes are usually not purified and are marketed as concentrated liquid or granulated products with specified enzyme lives. Enzymes used for

diagnostic or recombinant technology or in the fine chemical industries need to be highly purified products.

The industrial enzyme market is frequently segmented on the basis of applications, rather than the nature or classes of enzymes as enzymologists classify. Application sectors have been classified in different major sectors with respect to applications of enzymes.

1. **Food enzymes**
    a. Enzymes for starch processing (amylases for production of glucose)
    b. Sweetener production (glucose isomerase for fructose production)
    c. Bakery products (xylanase, α -amylase, glucose oxidase for dough conditioning, dough quality, loaf volume)
    d. Dairy product (rennin, lactase for milk coagulation and hydrolysis of lactose)
    e. Fruit juice (pectinase, cellulase, xylanase for juice clarification, juice extraction)
    f. Wine making (glucanase and papain for haze clearance)
2. **Enzymes for technological applications**
    a. Detergent enzymes (proteinase)
    b. Enzyme for textile (cellulase and laccase for microfibril removal and for improving brightness of cloth)
    c. Enzymes for leather processing (protease, lipase)
    d. Enzymes for paper and pulp processing (xylanase)
    e. Enzymes of analytical use such as:
        i. Uric acid by uricase
        ii. Ethyl alcohol by alcohol dehydrogenase
        iii. Ammonia by L-glutamate dehydrogenase
        iv. Cholesterol by cholesterol oxidase
        v. Glucose by glucose oxidase
        vi. Urea by urease.
3. **Enzymes for animal feed**
    a. Xylanase for fiber solubility
    b. Phytase for removal of phosphate
4. **Enzymes for medical applications**
    a. Digestive enzymes: Pancreatic enzymes, mammalian protease (pepsin) plant proteases (Bromelain, papain), fungal amylases.
    b. Enzymes with potential therapeutic applications
        i. Asparaginase and glutaminase hydrolyzing L- aspargine and L-glutamine to aspartic and glutamic acids respectively in the treatment of leukemia.
        ii. Urokinase and streptokinase (plasminogen to plasmin) for dissolving blood clot in heart attack.
        iii. Penicillinase for hydrolyzing penicillin during acute penicillin allergy.
        iv. Hyaluronidase for hydrolyzing hyaluronate in heart attack
        v. Collaginase for hydrolyzing collagen in skin cancer
        vi. Uricase for oxidizing uric acid in gout
5. **Enzymes for clinical and diagnostic applications**
    a. Enzyme linked immunosorbent Assay (ELISA): Enzymes used are: peroxidase, alkaline phosphatase, ß- galactosidase
    b. Enzyme multiplied immunoassay technique (EMIT): Enzymes used are lysozyme and malate dehydrogenase

c. Enzymatic analysis of blood constituents: Glucose, uric acid, urea, cholesterol, pyruvate, lactate, triglyceride etc.

Immobilized enzymes

Enzymes accelerate different chemical reactions with high specificity and are not permanently modified by their participation in reactions. But enzymes are costlier than chemical catalysts, in general, and cost effectiveness of enzyme-based processes could be reached by the repeated use of enzymes. But enzymes remain in solution with products and it is not possible to recover them easily from the reaction mixture. If they are made insoluble or stationary in active forms, repeated use of an enzyme becomes possible. Immobilization is the process by which an enzyme is made insoluble or stationary with the retention of full or substantial activity. Immobilization is also localization or confinement of enzymes during a process, which permits separation of the enzyme from substrate and product for its repeated use. The use of insoluble form of an enzyme in a process offers a number of advantages such as:

a) Repeated use of the same enzyme as far as practicable,
b) Ability to terminate reaction at any stage by the removal of insoluble enzyme,
c) Recovery of enzyme free product
d) General improvement of enzyme stability.

Techniques used for the immobilization of enzyme activity may be classified as:

a) Physical adsorption of enzyme on inert insoluble carrier
b) Fixing of enzyme on insoluble support by covalent binding
c) Entrapment of enzyme activity in polymerized gel
d) Insolubilization of enzyme by cross-linking with bifunctional reagent.

However, it is understandable that some changes of physical and chemical properties of immobilized enzymes may take place because of the development of new microenvironment around enzyme by the supporting matrix. The changes are usually expressed to various extents by the altered stability and kinetic parameters of the enzymes. Stability of the enzyme either increases or decreases on immobilization depending on the effect of the microenvironment on stability and denaturation of the enzyme. Enzymes attached to inorganic matrices were found to be more stable than those attached to organic polymers. Specific activity of an enzyme usually decreases upon immobilization, possibly due to partial denaturation of protein depending on the process of coupling between enzyme and matrix. The presence of electric charge of the matrix affects pH optima of enzymes and the insoluble matrix limits diffusion of high molecular substrates due to steric hindrance.

In the immobilization of an enzyme, it is most important to select the method of attachment, which will not affect or interfere with the substrate-binding site of the enzyme. Considerable knowledge of the active site of the enzyme is essential and any possible interaction with binding site is avoided. Active site of the enzyme is sometimes protected during attachment and freed later. Use of substrate or competitive inhibitor to protect the active site was found to be useful in some cases. Enzyme as biocatalyst can be immobilized using either purified/semi-purified enzyme (without undesired contaminant activities) or whole cells or sub-cellular components. Most of the enzymes used in industry are microbial extracellular enzymes, which can be isolated more easily from fermented broth as crude enzyme. Extra cellular enzymes are generally more stable than intracellular enzymes against environmental stress. The cost of enzyme is kept low by the development of fermentation with high enzyme

yielding strain. Lengthy and expensive methods of enzyme purification are avoided. Immobilization of whole cell containing the enzyme activity is highly cost effective and the process has some advantages. It preserves the natural environment of the enzyme and loss of activity of immobilized enzyme for each cycle of operation is generally lower than that of free enzyme. Whole cells also provide a number of catalysts present in the cell, if required in a process. But major disadvantages are limitations on the diffusion of substrate to and product from cell and possibility of unwanted side-reactions in presence of other enzyme in whole cell. Viable and non-viable cells are both immobilized. For single step processes, non-viable cells are used and cells are permeabilized by various physical and chemical treatments. Permeabilization causes diffusion of substrate or product through cell membrane but removes most of the small molecular weight compounds including co-factors from cells. Such non-growing cells are highly useful and economical source of intracellular enzymes for simple bioconversions requiring no regenerated co-factors for activity. A number of techniques have been developed for permeabilization of cells such as treatment of yeast or bacteria with toluene or sonication of cells etc.

There is no best-known method for the immobilization of any specific enzyme. The support, the enzyme, the substrate and technique, are all involved in the development of an effective process. The support material should be non-toxic, low cost, maximum biocatalyst loading capacity and with good flow character and operational durability.

Various methods used for immobilization of enzymes

In the process of immobilization by binding of enzyme to an inert carrier or cross-linking of enzyme to form an insoluble aggregate, the three dimensional structure of the active site, substrate and catalytic specificities of the enzyme must be kept unchanged during the process of immobilization. Chemical activities, free functional groups of the protein molecule such as α-,β-, γ-, carboxyl groups, free amino groups, thiol, hydroxyl, imidazole groups of amino acids are exploited if they are not involved in formation of catalytic site of the enzyme.

Adsorption on solid support

Adsorption of enzyme on solid surface (through Van der waals force or ionic interaction) has advantages because of its simplicity and non-involvement of any chemical reaction, which might affect catalytic activity of the enzyme. But this binding process is a reversible one and desorption of the enzyme becomes significant in presence of high substrate and ionic strength of the medium. Various inorganic matrixes such as alumina, activated carbon, clay, glass, controlled pore glass bead, hydroxy-apatide etc. are used in the immobilization process. Whole cells also could be immobilized using porous brick (yeast), silica (yeast), celite, diatomaceous earth Kieselguhr (mycelial cell), wood chips (yeast) etc. Hydrous metal oxides like those of titanium, zirconium, iron, tin, vanadium are capable of forming active enzyme insoluble complex.

Enzymes may be physically attached to insoluble matrix by ionic interaction. Ion exchange resins, e.g. DEAE-cellulose, DEAE-sephadex, Carboxymethylcellulose have been used as support media. However this binding is very sensitive to changes of pH and ionic strength of the medium.

Covalent coupling of enzyme on activated matrix

Covalent binding of the enzyme is more stable than adsorption attachment and enzyme is not leached out easily by changes of pH or at high salt or substrate concentrations. But the process, in general, is more drastic and immobilization is mostly associated with some loss of enzyme activity. Among the available reactive groups of amino acids, free amino group of protein is predominantly chosen, although coupling through other functional groups is also known.

A few chemical reactions commonly used for the formation of covalent bonds between enzyme and matrices are as follows:

i) The widely used method for immobilization of enzyme on polysaccharide matrix is cynogen bromide activation of matrix. CNBr interacts with –OH groups of the polysaccharide positioned side by side, to produce immidocarbonate derivative. The activated polysaccharide interacts spontaneously with free amino group of amino acid forming covalent bond.

ii) Diazonium derivative of insoluble career (*p*- aminobenzyl cellulose) is used for coupling with phenolic imidazole groups of proteins.

iii) Hydrazide derivative of insoluble matrix is used for coupling
Methyl ester of carboxymethyl cellulose + Hydrazine = hydrazide derivative→ HNO2 → Azide derivative + NH_2-Enzyme → Immobilization

iv) Immobilization on synthetic matrix may be exemplified as
Polyaminopolystyrene + $CoCl_2$→Isocyanate derivative of polystyrene → NH2-Enzyme (immobilization)

v) Glutaraldehyde is a useful reagent for the formation of covalent linkage between enzyme and matrix. Enzyme could be linked to aminoethyl cellulose or amino alklylated porous glass through this bifunctional reagent,
CHO-CH_2-CH_2-CH_2-CHO. Bis-diazobenzidine also acts as a bifunctional reagent for forming covalent linkage between enzyme and activated matrix.

Entrapment of enzymes and microbial cells

i. Calcium alginate

Alginic acid is a co polymer of β-mannouronic acid ά-L-Glucuronic acid linked by (1→4) glycosidic linkages. The polysaccharide is a constituent of marine algae. Alginate solution produces gel in presence of calcium ion (sensitive to PO_4^{2-}). Systems reported such as:

Entrapped cells of *Alcaligenes eutrophus* in calcium alginate for the production of H_2 from formate.

Production of isomaltulose from sucrose by *Erwinia rhapontici*

Photosynthesis by immobilized algae.

ii. Carragenan

It is a polysaccharide obtained from red sea algae. It is a linear sulphated polysaccharide containing D-Galactose, 3, 6-anhydro-D-Galactose and their sulphate ester derivative. Among carrageenans, Ḱ-carrageenan is insoluble in cold water as potassium salt but is sensitive to Na^+. Some of immobilized systems are:

Production of aspartic acid by entrapped Escherichia *coli.*

Production of ethanol by entrapped Zymomonas *mobilis.*

iii. Agarose

The polysaccharide is obtained form marine plant. It contains alternating 1, 3 linked D-Galactopyranase and 1, 4- linked 3, 6-anhydro-L- Galactopyranose (Linear polymer).

Entrapment of *Escherichia coli* in agar beads for the production of hydrogen from formate.

iv. Gelatin

Gelatin is an abundant, inexpensive and safe matrix for immobilization of microbial cells. The protein character, high hydrophilicity and good swelling properties of the matrix are highly favorable for immobilization process. After immobilization, gel is hardened by formaldehyde. A large number of enzyme activities of whole cells were immobilized (e.g. β-glucosidase, urease, invertase and acid phosphatase).

v. Polyacrylamide Gel

Aqueous solutions of acrylamide, N′- N′-ethylene bisacrylamide are polymerized in presence of suitable initiator and accelerator. Enzyme is entrapped within lattice of polyacrylamide gel. [11]β-Hydroxylation of cortexolone is done by *Curvularia lunate*, and *Aspergillus niger.*

vi. Cross-linked pre-polymerized polyacrylamide

Hydrazide gel partially substituted (acyl-hydrazide) linear polyacrylamide cross linked by glyoxal.

Cephalosporin production by *Steptomyces claruligerus.*

Δ'-Steroid reduction by *Mycobacterium species.*

Glucose to ethanol by *Sacchromyces cerevisiae*

***vii.* Photo cross-linkable resin prepolymers and urethane**

Prepolymer methods use synthetic resin prepolymers, which are photo- cross-linkable resin, polymerize on illumination with UV light. Polyethyleneglycol dimethacrylate, polybutadiene, maleic polybutadiene and polypropylene glycol are used for the purpose. Hydrophobic photo-cross linkable prepolymer (ENT-2000, ENT-4000, ENT-6000, PEGN-1000 / 2000 / 4000) have been used for entrapping sucrase, glucose isomerase, lipase, amino acid deacylase activities.

Water miscible urethane prepolymer (PU) contains isocyanate functional groups at both termini and molecules react with each other in the presence of water. Peroxisome from methanol oxidizing yeast has been successfully immobilized by polyurethane prepolymer.

Micro capsulation

Synthetic microcapsules are prepared by the use of hydrophilic and hydrophobic monomers. Enzyme is added with hydrophilic monomer in water and mixed with hydrophobic monomer emulsion in organic solvent. Microcapsules of 10-100 μm diameters are formed with enzyme present in water within. Enzymes are also capsulated in liposomes. Liposomes are lipid membrane with a water droplet inside. They are usually prepared from phosphatidylcholine and cholesterol.

Cross linking of enzymes

Same enzyme molecules may be cross-linked by bifunctional reagents such as glutaraldehyde and bis-benzidine. Sufficient cross-linking, occurring at high enzyme concentration makes the enzyme insoluble. However the technique is not much preferred, as it requires large quantities of enzyme and insolubilization is associated with some loss of enzyme activity.

Commercial processes in operation

Among the large number of processes reported for the immobilization of commercial enzymes, immobilized enzymes operated successfully in large-scale commercial process are:

a) Isomerisation of glucose for fructose production.
b) Aminoacylase system for amino acid production.
c) Lactase for hydrolysis of whey lactose.

Processes for producing high fructose corn syrup using glucose isomerase use both granules or amorphous or fibrous form of immobilized enzymes with productivities ranging from 1000 - 9000 Kg of 42 % fructose syrup /kg enzyme. The different immobilization procedures used include, DEAE cellulose adsorbed enzyme (*Streptomyces* enzyme), cross-linked lysed cell (*Bacillus coagulans*), gelatin entrapped and glutaraldehyde cross-linked whole cells (*Actinoplanes*), inorganic carrier adsorbed cells (*Streptomyces spp*) etc.

Lactase hydrolyzing lactose into glucose and galactose has a number of applications, particularly in utilizing lactose present in appreciable concentration in whey. In this respect, a large number of immobilization techniques were reported from time to time. Examples include covalent attachment of lactase to glass, collagen, sepharose, entrapment of enzyme in hollow fiber, polyacrylamide gel, and cellulose acetate and in ionizing radiation induced polymers of acrylate and methyacrylate. Lactase immobilized by binding on cellulose sheets was found to be stable for several months. *Aspergillus* lactase immobilized on controlled pore glass or titanium was reported to be available at a very low cost.

Commercial process using aminoacylase system for deacylating acetyl DL– Methionine operated commercially using different immobilized systems. The enzyme attached to DEAE-sephadex by ionic interaction, enzyme covalently bound to iodoacetyl cellulose, enzymes entrapped in polyacrylamide gel were used in different commercial processes.

Production of glucose from starch

Starch is synthesized naturally by a variety of plants but some plants produce a high amount of starch such as corn, potato, rice, sorghum, wheat and cassava. Starch contains two types of polysaccharides both containing glucose. An unbranched single chain polymer of 500-2000 or more glucose residues, linked through α-1, 4- glucosidic linkages is called *amylose*. The other fraction called *amylopectin* is branched where branch α- 1, 4-linked glucose chains are linked through α-1, 6- glucosidic linkages to the main chain. The degree of branching in amylopectin is about one per twenty-five residues of unbranched fragment of the main chain.

Starch in general is not soluble in water. It is partially crystalline and quite compact due to intra and inter-molecular hydrogen bonding. When an aqueous suspension of starch is heated at higher temperature, hydrogen bond gradually become weaker and water molecules enter into the starch granules. This process, known as gelatinization, makes starch susceptible to the action of enzyme. Gelatinization is usually accomplished at 90-100^{o} C in water. Addition of alkali to starch suspension and neutralization of alkali also causes gelatinization of starch at room temperature.

Corn Syrup

The knowledge that starch yields a sweet substance when heated with acid was available to chemists two centuries back. Corn emerged as the best source of starch because of its low cost, high availability and long storage life. Acid hydrolysis of cornstarch to produce 42 dextrose equivalent (DE) syrup was commercialized in early 20th century but the product could not compete with sucrose in terms of sweetening power or taste. During 1940-60, the discovery and isolation of amylases from microorganisms led to the development of a number of processes for the production of corn syrup. Various enzymatic hydrolysis protocols were introduced for the production of corn syrup in different food processing industries. Subsequently crystalline dextrose was obtained at 95% yield from cornstarch using amyloglucosidase along with bacterial and fungal α-amylases. The corn hydrolysed products known as "corn sweeteners" are sold on the basis of reducing sugar content or dextrose equivlent (DE). Higher DE value indicates higher degree of hydrolysis. The hydrolyzed product dextrose however remains contaminated with short or medium sized oligosaccharides and their presence lowers the DE of dextrose from 100 to various values. Different types of corn syrup have DE from 20 - 99.5, syrups with DE lower than 20 are usually called as malto-dextrin syrups.

Enzymatic hydrolysis of starch

Amylases are the enzymes, which hydrolyse starch to different extents. Enzymes hydrolyzing starch are produced by a wide variety of living beings, including humans. Human saliva and pancreatic secretions contain large amount of α- amylase for starch digestion. However major classes of α- amylases are produced by microbial fermentation. The important enzymes used in starch- saccharification process are α-amylase (EC 3.2.1.1), β- amylase (EC 3.2.1.2), amyloglucoside or glucoamyalse or γ-amylase (EC 3.2.1.3), pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68). In the hydrolysis of starch, enzymes should specifically hydrolyze both α- 1,4 and α- 1,6 linked glucose molecules in starch.

α- Amylase (α-1,4 glucan-4-glucanohydrolase)

The enzyme is an endo- glucanase, hydrolyzing α-1,4- glucosidic linkages in the interior of the starch molecule. The action of the enzyme is stopped when α- 1,6-glucosidic branch linkages are reached. The hydrolysis of amylose fraction of starch by a α- amylase results in the production of oligosaccharides or dextrins from 1,4-linked glucosidic chain in first phase. Dextrins are subsequently hydrolyzed into maltose and glucose. When amylopectin is the substrate, the hydrolysis products consist of a mixture of unbranched and branched oligosaccharides in which α-1,6-bonds are present The enzymes produced by different groups of bacteria and fungi are classified into liquefying and saccharifying amylases. Liquefying amylases quickly lower the viscosity of gelatinized starch by converting starch into dextrins but slowly hydrolyse dextrin into maltose. Saccharifying amylase, on the other hand, slowly lowers the viscosity of starch by dextrinization but quickly produces maltose and glucose from dextrins.

A large number of *Bacillus* species and fungi like *Aspergillus, Penicillium, Mucor* and *Rhizopus* produce saccharifying and liquefying α-amylases. Amylases from *A.oryzae, A. niger*, *Bacillus amyloliquifaciens*, *Bacillus licheniformis* are commercially produced.

β - Amylase (α, 1,4- glucan maltohydrolase)

This enzyme is an exo-enzyme that liberates maltose from linear non-reducing end of the polysaccharide. When β - amylase acts on amylase, it yields maltose quantitatively. But amylopectin is digested by the enzyme to yield maltose and a highly branched dextrin as the enzyme could act on α-1,4-linkages of amylopectin upto 2-3 glucose residues away from the branched 1,6-linkage. Thus, starch which contains both amylase and amylopectin, is hydrolysed by β - amylase into maltose and a highly branched core of amylopectin. Microbial producers of the enzyme are *Bacillus polymyxa, B.megaterium, Streptomyces spps, Pseudomonas spps, Rhizopus japanicus*. Bacterial enzymes are mostly active near pH 7.0 and do not require Ca^{2+} for optimal activity.

Amyloglucoisdase (α-1,4- glucan-glucohydrolases)

The enzyme amyloglucosidase or glucoamylase is an exo-enzyme that liberates α-1,4-linked glucose residues consecutively from non-reducing end of the starch molecule. The enzyme can hydrolyse terminal α-1,6-glucosidic linkages but much slower than α-1,4-linkages. Activity of the enzyme is lowest on maltose and increasingly higher with the increase of oligosaccharide sizes upto 5-6 glucose units.

Fungi are most active producers of amyloglucosidases. Enzymes are obtained from *Apergillus niger, A.oryzae, A.awamori*, and different strains of *Rhizopus.* Amyloglucosidses are frequently produced as isoenzymes by a single strain and all of them are not equally active on cornstarch.

1-6 Glucoside splitting enzymes

Enzymes capable of hydrolyzing α-1,6-branching of amylopectin are very important in the saccharification of starch into glucoses. Enzyme hydrolyzing amylopectin directly are pullulanase (EC 3.2.1.41) and isoamyalse (EC 3.2.1.68). Addition of pullulanases during enzymatic hydrolysis of starch by α amylase/ β amylase has a strong synergistic activity on the yield of glucose from starch. Isoamylases are produced by *Bacillus, Serratia*, and *Pseudomonas* spps. Pullulanase is capable of hydrolyzing both pectin and α -glucan microbial polysaccharides including pullulan, but isoamylase acts only on pectin. Pullulanases are produced by bacteria belonging to the genus *Aerobacter, Bacillus, Pseudomonas, Streptococcus*, etc.

The manufacture of dextrose from starch

The process involves the use of thermostable amylase and amyloglucosidase (and pullulanase) for hydrolysis of starch into dextrose monomers at yields close to quantitative. Gelatinized starch is liquefied and dextrinised by thermostable α-amylase. In a typical process this step is carried out in two stages with a holding time of 5-8 minutes at a temperature of 104 ^{o} C -107 ^{o} C followed by for 90-120 minutes at 94 ^{o} C - 97 ^{o} C. The gelatinized starch slurry contains suitable does of thermostable α amylase. The product obtained is soluble dextrin, a mixture of average DE of 10-15. To minimize colour formation due to Maillard reaction, under high temperature the protein content of the slurry is kept low and reaction is carried out at pH 6.0-6.5. The dextrin mixture is hydrolysed by

amyloglucosdiase into dextrose. A number of factors determine the final yield of dextrose, such as initial DE of substrate, pH temperature, concentration of solid, amyloglucosidase dose. In a typical process 94-96 % dextrose is obtained by amyloglucosidase treatment of dextrin syrup (30-35 % dry weight) at temperature of 60 ^{o}C and pH 4-4.4 for 65-75 hrs.

Enzymatic hydrolysis of cellulose into glucose

The huge plant-biomass, produced every day on earth due to photosynthesis contains cellulose as the major component. Microbial cellulose utilization, on the other hand, is responsible for largest return of the fixed carbon to the biosphere. Cellulose utilizing microorganisms present in soil and in the guts of animals play an important role in completing the carbon cycle on the earth. Cellulose degradation by microorganisms has a major role in anaerobic digestion, composting and for the supply of dietary protein by ruminants.

Cellulose is present in plant biomass in the range of approximately 35 – 50% of plant dry weight. In nature, pure cellulose is obtained from a few sources like cotton, bacterial cellulose etc. In plants, cellulose fibers are embedded in a matrix containing hemicellulose and lignin, which comprise 20 to 35% and 5 – 30% of plant dry weight. The composition of the complex termed as 'lignocellulosics' vary with plant cell type and also with the maturity of the plant. Cellulose is a linear polymer of D-glucopyranose joined together by β-1, 4-glycosidic linkages. Cellulose is synthesized as linear chains, which undergo self-assembly to produce cellulose fibers. Cellulose is initially assembled in elementary fibrils, which are further packed into larger units called micro fibrils, which are finally assembled to cellulose fibers. Hydrogen bonds and van der waals forces act between adjacent cellulose molecules giving rise to the parallel alignment and crystalline structure of cellulose. The extensive intra- and inter-chain hydrogen bonding produces straight, stable fibers of high tensile strength. A crystalline structure is a structure where all atoms in the molecule are present in fixed positions relative to each other. Thus crystalline cellulose prevents entry not only of enzyme but also of small molecules due to its extremely tight structure. But this crystalline structure is not uniformly distributed in fiber structure. There are many non-crystalline amorphous regions arising out of twists and other structural variations. These amorphous regions allow penetration of large molecules including cellulolytic enzymes.

Enzymatic hydrolysis of lignocellulose is more complex than that of pure cellulose. Diverse arrangement of cellulose fibers in different plant cells and presence of hemicellulose and lignin makes cellulose hydrolysis difficult.

Cellulose degrading ability is widely distributed amongst different bacterial genera and in many fungal groups. Cellulolytic bacteria include anaerobic Gram positive *Clostridium, Huminococcus*, Gram-negative *Butyrovibrio, Acetivibrio* and aerobic Gram positive *Cellulomonas, Thermobifida*.

Fungi are better known microorganisms decomposing cellulosic substrates; several species of anaerobic fungi and aerobic fungi including *Ascomycete, Basidiomycetes* and *Deuteromycetes* produce cellulolytic enzymes. Cellulolytic enzymes from the genera *Trichoderma, Aspergillus, Penicillum, Fusarium, Geotrichum etc.* have been studied in details.

The hydrolysis of cellulose into its monomeric component glucose, involves the participation of a number of enzymes, collectively known as cellulolytic enzymes or cellulases as such.

During the past two decades, extensive biochemical studies have been carried out on cellulase enzymes from aerobic and anaerobic bacteria and fungi. Three major types of enzyme activities identified as components of cellulase enzyme systems are:

i) 1,4- β-D-glucan-4-glucanohydrolase (Endoglucanase, EC 3.2.1.4)
ii) 1,4- β -D-glucan glucanohydrolase (Exo-glucanase, EC 3.2.1.74)
1, 4- β -D-glucan cellobiohydrolase (Exo-glucanase E.C.3.2.1.91)
iii) β -glucosidase or β -glucoside glucohydrolase (EC 3.2.1.21).

In the hydrolytic process, the mechanism of enzyme action was suggested to be as follows. Endoglucanases cut at internal amorphous sites of cellulose causing release of oligosaccharides of various chain lengths. Exo-glucanases act progressively on cellulose oligosaccharide chains, and liberate either glucose (glucanohydrolase) or cellobiose (cellobiohydrolase) as major products. Finally, β-glucoside hydrolyses soluble cellodextrins and cellobiose into glucose. A general property of most cellulases is their molecular structure, which consiste of a catalytic domain and a carbohydrate binding domain (CBD). CBD facilitates cellulose hydrolysis by bringing the catalytic domain of the enzyme close to the surface of insoluble cellulose substrate.

Cellulase systems are not simply mixture of these three enzymes, but an efficient combination of enzymes, which act, in a coordinated fashion to hydrolyze cellulose into glucose efficiently. Filamentous fungi and actinomycetes have the ability to penetrate cellulosic substrate by the elongation of mycelia and are capable of releasing enzyme on the substrate. These enzyme systems with or without CBM are called 'non-complexed cellulose systems'. Anaerobic bacteria, which cannot penetrate cellulosic substrate, produce 'complexed cellulase system' or cellulosome.

Non-complexed cellulase system

The cellulase system of aerobic filamentous fungus *Trichoderma reesei* was studied most extensively, followed by that of *Fumicola insolens* and of aerobic actinomycete of genus *Cellulomonas*. *T. reesei* produces at least five endoglucanases (EG I-V), two exo-glucanase (CBH I and CBH II) and two β -glucosidases. Exo-glucanase II and I preferentially attack microcrystalline cellulose from reducing and non-reducing ends of the polysaccharide. They are the major components of cellulase protein of *T.reesei*. The specific role of five individual endo-glucanases is not known clearly, but synergism between *endo*- and *exo*-glucanase has been observed for each enzyme. However, cellobiose the major end product inhibits activities of both *exo*- and *endo*-glucanase. The β-glucosidase produced by *T.reesei* hydrolyses cellobiase and small oligosaccharides to glucose. Both enzymes are present mostly in the cell bound form and little in the supernatant. Cellulase system of *H. insolens* is similar to that of *T. reesei* having two cellobiohydrolases, CBH I and II and five *endo*-glucanases. Cellulase system of cellulomonas is, however, slightly different from that of *T.reesei*. It contains one exo-glucanase and six *endo*-glucanases. However, each enzyme resembles that of *T. reesei* and has CBM.

Complexed cellulase system

Anaerobic microorganisms produce complexed cellulase system present in 'cellulosome' containing both cellulolytic and non-cellulolytic enzyme activities. Cellulosome is produced as protuberance on the cell wall of anaerobic cellulolytic bacteria like different strains of

Clostridia, e.g. *C.thermocellum, C.cellulolyticum, C.cellulovorans, C.josui* etc. and rumen bacteria *Ruminococcus*.

Cellulosome contains a large non-catalytic scaffolding (CipA) protein of 197 KDa size, which is anchored to the cell wall of bacteria. About 22 catalytic proteins are also present in cellulosome. Identified activities are those of nine *endo*-glucanases, four *exo*-glucanases, five xylanases, one chitinase and one lichenase. Cellulosomes are highly glycosylated and are stable protein complexes of 2 to 16 Mda, resistant to protease action.

Acid hydrolysis of cellulose for production of glucose

The process for the production of glucose by acid hydrolysis of cellulose has many drawbacks. Since hydrolysis of cellulose is to be carried out at high temperature where damage of equipments at high acidity is quick. So cost of hydrolysis equipment is very high also the sugar is spontaneously degraded in acid environment when released. Additional steps of acid neutralization of hydrolyzed product and purification of product from hydrolysate are also required to be included in the process. A few plants were operated in USA and Europe before World War II. In the slow acid hydrolysis process, dilute acid was percolated down through packed bed charged with wood chips with continuous removal of sugar solution from the bottom. The reactors were heated with steam at 50 psi. The hydrolysis was continued for 2-4 hour and acid was neutralized with lime. However yield of sugar from cellulose was never higher than 40%. Since hydrolyzed liquor was a dilute sugar solution, recovery of alcohol by fermentation was also costly. Acid hydrolysis for the production of more concentrated solution was also attempted using high-pressure superheated steam. But none of the processes were found to be economic for large-scale production of directly fermentable glucose from cellulose.

Enzymatic hydrolysis of cellulose into glucose

A wide variety of cellulosic substrates are easily available at a low cost, but the main problems associated with low biodegradability of lignocellulosic compounds are:

i) Cellulose is present mostly in crystalline form, which is relatively less accessible to hydrolysis.
ii) Low porosity, which does not allow the cellulolytic enzyme to gain access to the cellulose fibers.
iii) Cellulose micro fibrils are tightly surrounded by lignin, which acts as a cement between cellulose fibers. In addition, hemicellulose (xylan) and other structural polysaccharides form a physical barricade to enzyme action.

Pretreatment of cellulosic biomass

Milling of cellulose to 200-mesh increases enzymatic conversion of substrate to sugar by 60-70%. But milling is very energy and capital intensive. The degree of polymerization of cellulose also could be reduced by treatment with alkali, NH_3, or NO, all of which break the fibril hydrogen bond. Treatment of cellulose with ozone or cadoxen also lowers crystallinity of cellulose. High-pressure stem explosion converts the recalcitrant substrate into a hydrolysable substrate for cellulase. But all these pretreatment operations are costly and contribute largely to the cost of production of glucose from cellulosics.

In the commercial production of glucose from cellulose, cost of enzyme, however, is not favourable still today. Highest activity so far, has been reported for enzymes from

Trichoderma reesei - Rut C-30. Cellulolytic enzymes so far obtained from other microbial sources are also not available at an economic price. Although many pilot plant studies have been carried out, enzymatic hydrolysis of cellulose has not yet been operated on a commercial scale.

Lactase in the dairy industry

Lactose is present in milk at about 4.7% (w/v) concentration. Unfortunately, a large fraction of world's adult population shows lactose intolerance and has difficulty in consuming milk and dairy products. Severe tissue dehydrating diarrhea and death are also reported after feeding milk to lactose intolerant children and adults suffering from protein-calorie malnutrition. The availability of low lactose-milk is very important in food-aid programme. In cheese making, the supernatant left after coagulation of milk protein, contains appreciable amount of lactose and the whey cannot be easily disposed off into the environment because of high oxygen demand. On the other hand concentration of whey to syrup causes crystallization of lactose, giving a sandy texture.

Enzyme lactase, a β-galactosidase (EC 3.2.1.23), hydrolyses lactose to glucose and galactose. The hydrolysis of lactose in the milk prevents digestive problems of lactose intolerant population. Lactose hydrolysis has more advantages, it increases sweetness of resultant milk, and it improves scoop and creaminess of ice cream, yoghurt and frozen deserts. The disposal problem of cheese-whey may be managed by the hydrolysis of lactose, when product becomes four times sweeter and whey may be concentrated to microbiologically secured syrup (70%, w/v).

Although a number of microorganisms are known to produce lactase (β-galactosidase), enzymes from *Aspergillus niger, A. oryzae*, *Kluveromyces fragilis and K .lactis* are supposed to be safe as they have history of safe use for human consumption.

Hydrolysis of lactose is done either by free enzymes in batch process or by immobilized enzyme or by immobilized whole cell. Several commercial immobilized systems have been developed for large-scale operation. In Italy, industrial scale milk processing technology uses fibre-entrapped yeast lactase in a batch process.

Properties of lactase (temperature and pH optima) from different microorganisms determine their applications. Fungal lactases, which work in the acidic range of pH 2.5 – 4.5, are used for acid whey hydrolysis. Yeast and bacterial lactase in neutral pH 6 – 7.5 are suitable for milk and sweet whey hydrolysis. Another important property, determining the application of the enzyme is inhibition of enzyme activity by the product galactose. Enzyme more susceptible to inhibition by galactose is only operative in a dilute solution of whey (at low lactose level) in immobilized column.

Fructose production

Production of glucose syrup of high dextrose equivalent (DE) from corn became a very successful biotechnology by the use of amyloglucosidase in the saccharification process during 1940 – 50. But sweetness of D-glucose is less (70%) than that of sucrose on weight basis and lower solubility of glucose also posed problems. A commercial syrup containing 71% (w/v) of 97DE glucose has to be kept warm to avoid crystallization while syrup with lower concentrations of glucose is susceptible to microbial contamination. In this respect,

fructose consequently received attention as being a sugar with 30% more sweetness than sucrose and two times more soluble than glucose.

Conversion of glucose into fructose requires a single isomerization step. Although chemical isomerization of glucose under alkaline conditions is possible yield is low, reaction is very slow and large numbers of undesired byproducts accumulate in the reaction mixture. Enzymatic conversion of glucose into fructose was known earlier. During glycolysis, the conversion needs participation of three enzymes: hexokinase, phosphohexoisomerase, fructose-6-phosphatase and of ATP. The whole process was commercially unviable, because of unavailability of these enzymes as commercial products and the cost of ATP. The existence of any enzyme capable for isomerizing glucose into fructose was not known before 1950. An enzyme capable of isomerizing D-xylose into D-xylulose was first reported from bacteria. The enzyme initially designated as 'xylose isomerase' was found to catalyze conversion of α-D-glucopyranose into α-D-fructofuranose in presence of cobalt ion. Subsequently, a large number of glucose isomerases were studied onwards from different microorganisms and the name 'xylose isomerase' was replaced by 'glucose isomerase' as enzyme induction did not require xylose to be present as an inducer.

Glucose isomerase (EC 5.3.1.5) catalyses the isomerisation of glucose to fructose. The reaction is reversible and a mixture of fructose and glucose is always obtained by the action of the enzyme on glucose. Most glucose isomerses used today are D-xylose isomerase (Xylose →xylulose) which are active on D-glucose and also on D- ribose. The enzymes used in different industries usually have high temperature optima. Commercial glucose isomerase producers are *Bacillus coagulans, Streptomyces* and *Actinoplanes* species and *Flavobacterium arborescens*. Some bacteria like *Escherichia intermedia*, *Aerobacter aerogenes* also isomerise glucose through glucose phosphate isomerase enzyme. The commercial process for the production of glucose from fructose became possible only when enzyme was successfully immobilized. The enzymes are usually intracellular, and usually whole cells or broken cells are used as a source of the enzyme for immobilization.

In the modern process for the production of high fructose syrup, the basic process is enzymatic isomerisation of dextrose by immobilized glucose isomerase. But the process in total, includes many steps, like dextrose production (usually from corn starch), purification of dextrose syrup to make it suitable for feeding to glucose-isomerase immobilized column, isomerisation of dextrose to produce 42% fructose, refining of fructose and fractionation of the syrup for high fructose corn syrup production.

The most important factor in the use of corn dextrose syrup is the purity of the feedstock. The feedstock should have low color, low ash content and low level of impurities such as metal ions and proteins. The refining process includes use of series of filters to remove traces of particulate matter, decolourizarion and deionization of the filtrate respectively with activated carbon and by cation-anion exchange resins. Magnesium ion is added in the feedstock, which activates glucose isomerase system. Glucose isomersase enzyme is also protected by magnesium from inhibitory action of calcium ion, if present in the feed.

Many immobilized isomerase preparations are available commercially. But cost of the immobilized enzyme is an important factor. The quality of enzyme is judged with respect to longer half – life and higher initial activity.

The conversion of glucose to fructose from 94–96% dextrose attained equilibrium at 60^{o}C, when fructose level reached to 47-48%. But a long residence time of dextrose with enzyme is required to achieve the equilibrium. The conversion level is usually maintained at 42% of fructose with lower residence time. During the process, rate of conversion is proportional to the enzyme activity of the immobilized column. But enzyme activity decreases gradually with time during operation, which either lower fructose concentrate at fixed residence time, or increases residence time for targeted fructose concentration. In operation, a battery of columns is used in place of single one to maintain desired yield at optimum period of time. Another important feature in the process control is the operating temperature and pH. Usually process is conducted at 55^{o}C in pH range of 7.0 – 7.5. Enzyme lives are usually between 3-4 months and columns are generally replaced twice a year.

High fructose corn syrup

Commercial 42% fructose syrup produced frequently does not comply with the quality specifications demanded for the use in soft drinks. Fructose syrup containing more than 55% sucrose was essentially required for high quality soft drinks. The composition of the equilibrium mixture obtained during action of glucose isomerase, ultimately determines the percent of fructose in the reaction product. Thermodynamics of the system favors fructose production at elevated temperature. The enzyme reactor, which could operate at 95˚C, is likely to yield a reaction mixture containing 55% fructose. Water soluble organic solvents also bring similar effects on equilibrium mixture. But none of the processes were found to be economically viable. Attempts were subsequently made to isolate fructose-rich fraction by various fractionation methods suitable for commercial exploitation. Chromatography of fructose syrup on Zeolite or calcium salts of cation exchange resin yielded fructose-rich fraction (~90%). The fraction blended with 42% syrup yielded 55% fructose syrup suitable for use in soft drink. Other fractions eluted from ion exchange column, are rich in glucose and contain oligosaccharides. These fractions were treated with glucoamylase to obtain glucose syrup containing 20% solid.

Proteases in food processing

Proteases are produced commercially from plants, bacteria and fungi. Enzymes are generally grouped as alkaline, neutral and acid proteases according to their proffered pH range of activities.

Alkaline protease

Both bacteria and fungi produce alkaline proteases. Various *Bacillus* strains (*B.amyloliquifaciens, B.subtilis, B.megaterium*), Streptomyces strains (*S.fradiae, S.griseus, S.rectus*) and fungi such as *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus oryzae* are potential producers. Subtilisin carlsberg (*Bacillus licheniformis*) and subtilisin BPN (*B. amyloliquifaciens*) are produced in large scales. These enzymes are also called serine proteases, having serine at their active site. Proteases having higher temperature optima, good stability in presence of detergent formulations and working in alkaline range (pH 9-11) are produced commercially in large scale. Alkaline proteases of mammalian origin such as chymotrypsin and trypsin are also serine proteases, having optimum activity near pH 8.0. However these enzymes are structurally different from bacterial serine proteases.

Neutral protease

Neutral proteases are produced by most of the bacteria and fungi, which produce alkaline proteases. Neutral proteases are relatively less rigid than alkaline proteases, being active in narrow ranges of pH and temperature. Limited uses of these enzymes in the leather and bakery industries have been reported.

Acid proteases

We have known mammalian gastric enzyme pepsin and rennin of calf stomach for long time. Many fungi also produce acid proteases. Many enzymes are very similar to pepsin and rennin (chymosin). Enzymes produced from *Aspergillus* species, optimally acting in the pH range of 2-4 are used in many pharmaceutical preparations as digestive enzymes. Enzymes are used in industries for breaking gluten of wheat flour or hydrolyzing Soya protein.

Plant proteases (Bromelain and Papain) in food processing

Plant *proteases* such as papain from papaya fruit and bromelain from pineapple have been used in food processing for centuries. These enzymes are thiol proteases having essential cysteine residue in the active site.

Papain present in leaves and unripe fruit of *Carica papaya,* was used to tenderize meats from very ancient times. Papain is commercially produced from the latex of papaya fruit. The latex is obtained by cutting the skin of unripe papaya. Fruits are normally tapped thrice at about 4 – 7 days intervals. The flow of latex usually ceases 4 – 6 minutes after incision is made. The latex is collected and stored in polythene coated tightly filling boxes, kept under shade. During collection, precaution is taken to avoid contact of latex with heavy metals, such as iron or copper. All containers including knives, spoons and pots used are either made of plastic or stainless steel. Papain is potentially dangerous, it damages skin on hands under prolonged contact and sometimes causes allergic reaction. The crude papain is obtained by drying the latex. The method of drying largely determines the quality of papain. Crude papain has major use in meat industry for tenderization of meat. Brewing industries in Britain also use papain for chill- proofing beer. The latex of papaya contains many active proteinases; two major types, papain and chymopapain have been obtained in crystalline forms. Papain has a molecular weight of about 20.5 KDa and isoelectric point at pH 8.8. Papain acts over a wide pH range with optima near pH 6.0. Specificity of peptide bond hydrolysis of papain is similar to those of trypsin or chymotrypsin. It preferentially hydrolyses peptide bonds adjacent to arginine or lysine and tyrosine or phenylalanine. Papain is activated by a variety of substances like glutathione, cysteine, H_2S, HCN etc and it binds metal ions Cu^{2+} and Hg^{2+}.

Meat of older animals can be tenderized by injecting inactive papain (oxidized disulphide form) into the jugular vein of live animal before slaughter. After slaughtering, reducing conditions developed in the meat reactivate oxidized papain, which tenderizes the meat. But later, this practice was found to be inconvenient. Action of papain in slaughtered animals could not be controlled and quality of meat was found to deteriorate with time.

Bromelain is a glycoprotein of 33 KDa molecular weight present in pineapple juice and stem tissue. It has uses in beer clarification and tenderizing meat similar to papain. However the enzyme is costlier than papain and use of bromelain is comparatively low compared to that of papain.

Protease in cheese making

The use of rennet in cheese making is a very old technology known to mankind for several centuries. Rennet (bovine chymosin), obtained from the fourth stomach of unweaned calves has been used for the production of cheese as a milk-clotting agent. Rennet hydrolyses specific peptide linkage between phenylalanine and methionine residues of κ -casein protein present in milk. The κ -casein maintains the colloidal character of milk. In the protein molecule, N-terminal hydrophobic domain of κ-casein remains associated with insoluble α- and β-casein while negatively charged C-terminal interacts with water and all these interactions prevent casein micelles from growing large. Action of rennet hydrolyzing phenylalanine-methionine bond, separates the two domains with the release of hydrophobic para-κ-casein. Termination of the protective action of κ -casein causes coagulation milk to form curd. The coagulated protein is compressed and recovered as cheese. The enzymatic process is temperature dependent and requires presence of calcium ions. An efficient transformation of milk into gel depends on both the factors. Calf rennet, which contains chymosin and a small amount of pepsin, is a relatively expensive enzyme and has limited supply. Extensive research has been carried out to obtain rennet-like proteases from microorganisms. The major utility of rennet in cheese manufacturing is its proteolytic mode of action and its easy thermal inactivation during ripening of cheese. Presence of any residual protease was found to cause development of unwanted bitter off-flavors in cheese after ripening. Chymosin is a relatively unstable enzyme. Little activity of the enzyme remains during ripening of cheese. Many microorganisms are known to produce rennet-like proteases. *Rhizomucor pusillus, R. miehei, Aspergillus oryzae, Irpex latis* enzymes are used in cheese manufacture. Milk clotting enzyme from *R. pusillus* is obtained by the solid fermentation of the fungus on wheat bran. Enzymes from *R. michei* and *Endothis parasitica* are obtained from submerged fermentation. Microbial enzymes with lower thermostability are more suitable for cheese making. Loss of protease activity after milk clotting similar to chymosin, is appreciated. This protease is not likely to develop off-smell of cheese during ripening. An interesting example of chemical modification of the protease, successfully lowering its thermo stability was reported. Enzyme from *M. miehei* was treated with oxidizing agents like par acids or hydrogen peroxide to lower heat stability. The oxidation converts methionine residue of *M. miehei* enzyme to methionine sulphoxide and thermo stability of the enzyme was lowered by 10˚C. The treated enzyme was found to be very similar to chymosin, producing no off-flavor during ripening of cheese. Attempts were also made to clone chymosin into *Escherichia coli* or *Saccharomyces cerevisiae.* The calf pro-chymosin gene was expressed in *E. coli* as insoluble inclusion bodies from which prochymosin was recovered after denaturation. Enzyme produced by *Saccharomyces cerevisiae* containing cloned prochymosin gene mostly remained bound with cellular debris with only 20% in the soluble supernatant. Later chymosin was successfully cloned and expressed in *Kluveromyces lactis* and large-scale production of the enzyme was developed.

Microbial rennets from various microorganisms are marketed under different trade names. Although proteolytic activities of different fungal rennets are considerably different from that of chymosin, but acceptable cheeses are produced by the use of these enzymes.

Other minor uses

Heat labile fungal proteases are used in baking processes to hydrolyze gluten (protein fraction) present in flour. Weak-gluten flour is favored for biscuit manufacturing and unavailability of flour of that quality is managed by the addition of protease in dough

prepared from high-gluten flour. Proteases are also used for the recovery of protein from parts (bone) of animals and fish. Controlled hydrolysis of Soya protein by proteases is carried out to produce verities of hydrolysates with different new properties.

Enzymes in leather industry

One of the oldest industrial uses of enzyme activity is in leather processing. Before raw hide is transformed into leather, it undergoes a series of operations whereby leather making protein collagen present in hides and skins is freed or partially freed from non-collagenous constituents. Hides and skins contain fat as well as globular proteins, *viz.* albumin, globulin, mucoids and fibrous protein such as elastin, keratin and reticulin between collagen fibers. In industry, raw material is processed through a series of operations including soaking, liming, dehairing, deliming, bating, degreasing and pickling. In pre-tanning operations, skins and hides are subjected to a water soak, which cleans the raw material and loosens the hair. The conventional and most widely used method for dehairing is the treatment of soaked raw material with lime and sodium sulphide. Subsequent deliming is also done to remove adsorbed lime from the hide. Fat present in skins is usually removed with degreasing agents such as soluble lime soap, kerosene, chlorinated hydrocarbons or spirit. In the traditional processing, a large numbers of pollution causing chemicals, lime, sodium sulphide, and solvents are released in effluents, which are toxic and cause environmental pollution.

In addition of chemical treatment, an enzymatic treatment, known, as 'bating' is an essential step to obtain optimum results. During bating, the scud is loosened and many unwanted proteins are removed. Bating makes the grain surface of the finished leather clean, fine and glossy. The traditional practice of bating is an unhygienic process where uncontrolled fermentation of leather is conducted with manures of dog, pigeon or hen as sources of microorganisms. This process gives a desired character to the finished leather. No chemical process has been developed which can substitute the fermentation.

The leather industry has a major problem regarding industrial pollution due to the use of huge amounts of toxic chemicals and biological pollution by addition of unknown microbial load to the environment. Use of enzyme in pre-tanning processes appeared to be a viable alternative technology where pollution problems resulting from tannery effluent could be significantly regulated or restricted.

Enzymes in pre-tanning

Proteolytic enzyme is the most important enzyme used in pre-tanning process. Enzymes from plant, animals and microbial sources were known to leather industry for a long time. But development of enzyme-based process became bright with the success of production of enzymes at commercial level and availability of enzymatic formulations at cheap rate.

Animal proteases and microbial proteases from bacteria and fungi are used in leather industry. The physicochemical properties of the enzyme such as substrate specificity, temperature and pH stability and pH activity range are very important factors in the application of enzymes in different steps of pre-tanning operations. Microbial enzymes appeared to be ideal source of the proteases. Enzymes with wide pH range of activities could be produced economically from different microbes. Now various commercial enzyme preparations are available for use in leather industry.

Enzymes in soaking

Proteolytic enzyme combinations (*Aspergillus parasiticus, Aspergillus flavus, Bacillus subtilis, and Aspergillus awamori*) active in natural or alkaline pH ranges are usually used.

Enzymes in dehairing

Use of enzyme in dehairing is highly desired in leather procesing. It would eliminate the use of sodium sulphide, one of the most toxic chemicals with obnoxious odour. A large number of proteases from *A. flavus, A fumigatus, A. chraceus, A. effuses, Bacillus sp., Streptomyces sp.* have dehairing or hair loosening effects. Potential use of specific protease keratinase from *Streptomyces fradiae*, for dehairing was also indicated. Enzymes from fungal or bacterial sources are allowed to act at pH 10.0 for about 12 – 16 hours, and hair is removed by mechanical means.

Enzymes in bating

The process of bating is a method for softening hides by treating them in a warm infusion of animal dung. Deliming and proteolytic actions take place simultaneously in bating. A 'bate' usually contains a proteolytic enzyme, a carrier like wood flour and deliming agents like NH_4Cl or $(NH_4)_2SO_4$. Pancreatic enzymes, bacterial and fungal proteases of neutral and alkaline types are used in bating.

Proteases in detergent formulation

Protease used in detergent has the largest single market accounting for 25-30% of total enzyme sales. Proteases added to detergents remove protein from clothes soiled with blood, milk and other proteinaceous materials and thus work more efficiently than non- enzyme detergents. The idea of using protease in detergent goes back to the use of pancreatic extract by Roehm in 1913. But economic and technical successes were only achieved in 1960 with the availability of enzymes from bacteria. The basic requirements for detergent proteases are:

i) Availability at low cost from safe microbial source
ii) Capable of working in high alkaline pH of common detergent solution
iii) Sufficiently thermostable with higher temperature optimum
iv) Low or no allergic response for topical use

Interestingly the enzymes were produced from *Bacillus spps* from the beginning and alkaline proteins from these species represent the lead molecule for the protease group "Subtilisins'. Although subtilisins are classifed as serine proteases by their catalytic mechanism, but they clearly constitute a separate group in terms of their amino acid sequence and three-dimensional structure. Mainly two species, namely *Bacillus licheniformis* and *Bacillus amylolquifaciens* are large scale commercial detergent protease producers in the global market. In addition enzymes from other strains such as *B. clausit*, *B.lentus*, *B. alkalophilus*, *B. halodurans* etc are used in detergents worldwide.

All these enzymes are fairly non-specific endo proteases, preferentially hydrolyzing the carboxylic side of hydrophobic amino acid residue, but are also capable of hydrolyzing other peptide bonds. They quickly convert protein substrate into small readily soluble peptides. Detergent proteases under different trade names (Alcalase, Savinase, Esperase, Maxatase etc)

are produced commercially. The enzymes are active over a wide pH range up to pH 12 and temperature up to 60^{o}C.

In the fermentation of subtilisins, a large number of variables are manipulated to obtain high yield at low cost. Industrial production processes normally run as large scale using batch fermentations at a high cell density. The composition of fermentation media and downstream processing of the enzyme are not available in details because of company secrecy. Recent reports available for the production of bacillus subtilisins, indicated yields of enzyme in the range 20-28 gm/L of fermentation broth.

Proetin engineering with subtilisin has been tried over a long period to obtain new desired quality of subtilisin. Site directed mutagenesis with the replacement of a single amino acid residue adjacent to active serine residue resulted in the production of enzyme stable in presence of hydrogen peroxide. Hydrogen peroxide and peroxy acids are commonly used in detergents as bleaching agents. Extensive replacement of amino acid of subtilisin BPN (*B. licheniformis*) has been patented to obtain improved properties of the protease.

Earlier attempt at the production of detergent protease at commercial scale had problems due to the cases of development of allergic reaction of the workers by the enzyme dust. This was solved by the development of enzyme granules of average 0.5mm diameter. In this process enzyme in a mixture of inorganic salt and sugar as preservative, is present in an inner core with a protective colloid. This core is coated with polymers made from polyethylene glycol along with a hydrophilic binder. The granulated enzyme was found to be more resistant toward various additives like peroxide, optical brightener, and detergent present in the formulation.

Medical applications of enzymes

Therapeutically used enzymes are required in relatively small amounts, but at a very high degree of purity. Many of the enzymes are used topically and orally. A few are injected into blood circulation particularly in the treatment of life threatening disorders like cancer or heart attack.

In general enzymes are foreign proteins, antigenic in nature and elicit an immune response causing severe allergic reaction, particularly when used for longer periods. Antigenic property is modified in several ways, either by covalent modification or entrapment of enzyme within artificial liposomes, synthetic micro spheres and erythrocyte ghosts. The cost of the enzyme is sometimes found to be very high. Urokinase used for dissolving internal blood clots is prepared from human urine and is very costly compared to microbial enzymes. However genetic engineering is an useful technology, which is exploited for the production of the enzymes by microorganisms.

Digestive enzymes

Aging causes many individuals to suffer from the problem of indigestion. It has been established that after 40 years of age there is a gradual decrease in the production of digestive enzymes. In addition, poor eating habits like less chewing, late eating in the day and habits like excessive consumption of alcohol, refined carbohydrate and fat also cause inadequate production of digestive enzymes. All these problems could be minimized by the intake of digestive enzymes to improve the efficiency of digestion.

Pancreatic enzyme supplement derived from pork pancreas, is an excellent source of digestive enzymes, provides proteases, amylases and lipase. Although the enzyme supplement has a good record of success, its use has some limitations. Pancreatic enzymes are sensitive to low pH values and are destroyed by pepsin secreted from stomach. A pH sensitive coating has been applied on several pancreatic enzyme preparations, which dissolve only above pH 5.5-6.0. Thus enzyme activities are protected in low pH condition of the stomach. Supplementation of microbial enzymes particularly those produced by different species of *Aspergillus* has many advantages. They provide a large number of digestive enzymes such as proteases, amylase, lipase, lactases, maltase and invertase. They are normally stable over a wide range of pH compared to animal enzymes. They are active and functional for longer distances in the digestive tract. However microbial enzyme should be free from microbial residue and should be a clean and pure product.

Asparaginases

Asparaginase is an important chemotherapeutic agent used in the treatment of acute lymphoblastic leukemia and other lymphoid malignancies. The treatment is based on the fact that tumor cells are deficient in aspartate-ammonia ligase activity, which limits their ability to synthesize the normally non-essential amino acid L-Asparagine. During growth of tumor cells, L-Asparagine is drawn from body fluids. The enzyme is administered intravenously and it lowers aspargine levels in the blood stream. Asparginase does not affect normal cells as they produce sufficient L-Asparagine for their function. But concentration of extra cellular L-Asparagine is sufficiently lowered by Asparaginase, which restricts growth of tumor cells due to asparagine starvation. The major limitation in the asparginase therapy is the development of clinical hypersensitivity. Chemically modified Asparginase covalently bonded to monomethoxy polyethylene glycol has been found to be useful for the treatment of patients who are sensitive to the native enzyme.

Native Asparaginases, obtained from *Escherichia coli* or *Erwinia chrysanthemi*, are used either alone or in combination therapy for treatment.

Sports medicine

In sports medicine, therapeutic effects of proteases have been observed. Proteases support, enhance and regulate circulation and the immune system of the individuals, which helps to optimize workouts and muscle maintenance. Treatment of sports injuries with protease has shown good response in the recovery from inflammation and in speedy healing of bruises and swelling. It was reported that proteolytic enzymes obtained from plant sources are more effective than animal enzymes in wound healing of plastic surgery patients.

Streptokinase and Urokinase

In blood plasma, plasmin is an important serine protease, involved in dissolving fibrin clots. It is present in circulation as plasminogen which is activated to plasmin by a number of factors in tissue including tissue plasminogen activator, urokinase plasminogen activator etc.

Streptokinase is an extracelullar enzyme produced by β-hemolytic *Streptococcus.* It can exert fibrinolytic effects through activation of plasminogen. Plasmin in turn attacks fibrin to

degrade it into soluble products. Streptokinase is used as an effective and cheap clot dissolving enzyme drug during myocardial infarction and pulmonary embolism. It is given intravenously after the onset of a heart attack to dissolve blood clots in the arteries of the heart wall. Streptokinase is a single chain 47kDa protein secreted by various strains of *Streptococc*i. However, the enzyme obtained from *Streptococci* remains contaminated with a number of unwanted enzymes as present in the extra cellular medium with streptokinase. Streptokinase being a bacterial protein creates immunological problems (allergic reaction) when present in the circulation. Genetically engineered *E.coli* producing a large amount of intracellular streptokinase has been obtained. Attempt has also been made to obtain streptokinase from safer strains of *Bacillus subtilis* by genetic engineering.

Urokinase is also a medically important enzyme used as an anti blood-clotting drug for treatment of heart patients. The enzyme of mammalian origin is isolated from urine. The cost of purification of enzyme is subsequently much higher compared to that of streptokinase. The possibility for the commercial production of the enzyme from human kidney cell line in hollow fiber reactor is under trial.

Glucose oxidase electrode

Glucose oxidase (EC 1.1.34) catalyses the oxidation of β-D glucose to D glucono- 1-5 lactone and hydrogen peroxide when molecular oxygen acts as electron acceptor. D-glucono- 1, 5 lactone, however, hydrolyses spontaneously to gluconic acid. Although the enzyme is present in many fungi but that obtained from *Aspergillus niger* has been studied extensively. The enzyme is a dimeric 160KDa protein with one molecule of flavin adenine dinucleotide (FAD) present as cofactor per monomer. FAD is not bound covalently with the apo-enzyme; it can be extracted from enzyme under mild conditions and can be added to apo-enzyme to restore catalytic activity.

The enzyme exhibits a high degree of specificity for β –D-glucose. Michaelis constant of glucose oxidase with glucose is 20mM and with dioxygen is 1.25 mM. 2-deoxy-D-glucose, D-mannose and D-fructose are also oxidized, but at a much slower rates. Glucose oxidase can transfer electron to many artificial electron acceptors other than molecular oxygen. This activity has been used widely for the quantification of serum glucose by colorometric method. In the presence of peroxidase and an electron acceptor dye, oxidation of glucose is estimated by the development color of reduced dye.

Estimation of glucose is very important in a number of diverse situations. It is important in food industry, in fermentation processes for on line monitoring and most importantly as a clinical indicator of diabetics. Development of a portable easy analytical technique which can give immediate and reliable on-site results, is highly desired for the estimation of glucose.

Biosensor has been developed as the more convenient portable analytical tool for the purposes stated above. A biosensor, in general, is a self contained integrated device capable of providing specific quantitative and semi-quantative analytical informations using a biological recognition signal.. A catalytic biosensor (e.g. Glucose oxidase) is a kinetic device that measures steady state concentration of a transducer-detectable species formed or lost due to a biocatalytic reaction. The development of glucose biosensor became possible, as enthalpy change associated with the oxidation of glucose-by-glucose oxidase is large enough to be detected by a thermistor. It has been shown by the use of radiolabelled dioxygen that oxygen in hydrogen peroxide is derived from dissolved oxygen but not from water molecule.

In principle, the of estimation of glucose may be done on with respect to:

i) pH change due to acid production
ii) Oxygen consumption
iii) Hydrogen peroxide production

In the amperometric estimation based on oxygen consumption, the biosensor is consisted of a platinum electrode where oxygen is reduced with reference to Ag/AgCl reference electrode.

$$4Ag^0 + 4Cl^- \rightarrow 4AgCl + 4e^-$$
$$4e^- + 4H^+ + O_2 \rightarrow 2H_2O$$

When a potential (-0.6Volt) relative to Ag/AgCl is applied to platinum electrode a current proportional to the concentration of dissolved oxygen is produced. The rate of electrochemical reduction is dependent of oxygen concentration. Depletion of oxygen concentration by the action of glucose oxidase, is detected by the reduction of current between the electrodes. The biosensor is consisted of an electrode compartment containing a platinum cathode and an annular silver electrode, connected through a saturated solution of KCl. The electrode compartment is kept separated from enzyme compartment by a semipermeable thin plastic membrane permeable to oxygen only. This compartment contained immobilized glucose oxidase separated from test solution by a membrane permeable to sustrate and products only. Biosensors of diameters from 1cm to 0.25 mm are commercially available.

The alternative method for the development of glucose biosensor based on the production of hydrogen peroxide, is application of a potential (+ 0.68) directly to a platinum electrode, relative to Ag/AgCl electrode when following reaction takes place:

Pt anode $H_2O_2 \rightarrow O_2 + 2H^+ + e^-$
Ag Cathod $2AgCl + 2e^- \rightarrow 2Ag^0 + 2Cl^-$

The major problems of these biosensors are their dependence on dissolved oxygen concentration. Some mediator (Ferrocinium ion) which transfer electron directly to electrode is also used.

The biosensor used for the determination of unknown glucose concentration is made with glucose oxidase immobilized on the surface of polarographic oxygen electrode. In a typical process glucose oxidase is immobilized on a membrane by the use of bifunctional crosslinking reagent, glutaraldehyde. Biosensor based on glucose coating with glucose oxidase – immobilized gelatin was also developed. Enzyme has been immobilized onto gelatin by cross-linking with Chromium III acetate. The electrode has a response time of 60 seconds with detection limit of 0.25mM glucose. The accuracy was maintained till two months.

Suggested Readings

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