Microbial Enzymes

Course of Special Microbiology

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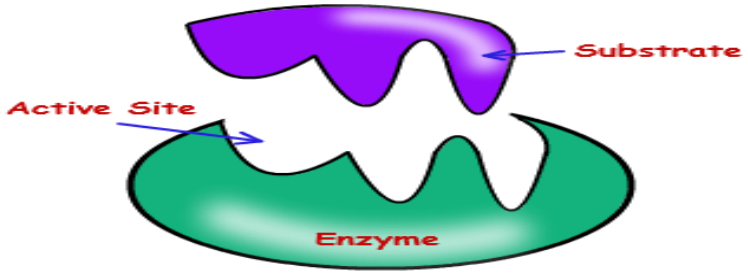
Content

Introduction

Enzymes are large biomolecules that are responsible for many chemical reactions that are necessary to sustain life. Enzyme is a protein molecule and are biological catalysts , Microorganisms are the primary source of enzymes, because they are cultured in large quantities in short span of time and genetic manipulations can be done on bacterial cells to enhance the enzyme production . In addition, the microbial enzymes have been paid more attention due to their active and stable nature than enzymes from plant and animal .

Most of the microorganisms are unable to grow and produce enzyme under harsh environments that cause toxicity to microorganisms. However, some microorganisms have undergone various adaptations enabling them to grow and produce enzymes under harsh conditions . Recently several lines of study have been initiated to isolate new bacterial and fungal strains from harsh environments such as extreme pH, temperature, salinity, heavy metal, and organic solvent for the production of different enzymes having the properties to yield higher.

Enzymes increase the rate of the reaction. Enzymes are specific, they function with only one reactant to produce specific products. Enzymes have a three-dimensional structure and they utilize organic molecules like biotin and inorganic molecules like metal ions (magnesium ions) for assistance in catalysis. Substrate is the reactant in an enzyme catalyzed reaction. The portion of the molecule that is responsible for catalytic action of enzyme is the active site.



Bacterial enzymes	
a-Amylase	*Bacillus*
b-Amylase	*Bacillus*
Asparaginase	*Escherichia coli*
Glucose isomeras	*Bacillus*
Penicillin amidase	*Bacillus*
Protease	*Bacillus*
Lipase	*E.coli*
Pullulanase	*Klebsiella*
Fungal enzymes	
a-Amylase	*Aspergillus*
Aminoacylase	*Aspergillus*
Glucoamylase	*Aspergillus*
Catalase	*Aspergillus*
Cellulase	*Trichoderma*
Dextranase	*Penicillium*
Glucose oxidase	*Aspergillus*
Lactase	*Aspergillus*
Lipase-	*Rhizopus*
Rennet	*Mucor miehei*
Pectinase	*Aspergillus*
Pectinlyase	*Aspergillus*
Protease	*Aspergillus*
Raffinase	*Mortierella*
Yeast enzymes	

Invertase	*Saccharomyces*
Lactase	*Kluyveromyces*
Lipase	*Candida*
Raffinase	*Saccharomyces*

Characteristics of enzymes are as follows:

- Enzymes possess great catalytic power.
- Enzymes are highly specific.
- Enzymes show varying degree of specificities.
- Absolute specificity where the enzymes react specifically with only one substrate.
- Stereo specificity is where the enzymes can detect the different optical isomers and react to only one type of isomer.
- Reaction specific enzymes, these enzymes as the name suggests reacts to specific reactions only.
- Group specific enzymes are those that catalyze a group of substances that contain specific substances.
- The enzyme activity can be controlled but the activity of the catalysts can not be controlled.
- All enzymes are proteins.
- Like the proteins, enzymes can be coagulated by alcohol, heat, concentrated acids and alkaline reagents.
- At higher temperatures the rate of the reaction is faster.
- The rate of the reaction invovlving an enzyme is high at the optimum temperature.
- Enzymes have an optimum pH range within which the enzymes function is at its peak.
- If the substrate shows deviations larger than the optimum temperature or pH, required by the enzyme to work, the enzymes do not function such conditions.

- Increase in the concentration of the reactants, and substrate the rate of the reaction increase until the enzyme will become saturated with the substrate; increase in the amount of enzyme, increases the rate of the reaction.
- Inorganic substances known as activators increase the activity of the enzyme.
- Inhibitors are substances that decrease the activity of the enzyme or inactivate it.

Nomenclature of enzymes

The current system of nomenclature of enzymes uses the name of the substrate or the type of the reaction involved, and ends with "-ase". Example:'Maltase'- substrate is maltose . 'Hydrolases'- reaction type is hydrolysis reaction. The International Union of Biochemistry and Molecular Biology have developed a nomenclature for enzymes, the EC numbers; each enzyme is described by a sequence of four numbers preceded by "EC", which stands for "Enzyme Commission". The first number broadly classifies the enzyme based on its mechanism.

Classification of enzymes

Enzymes are classified based on the reactions they catalyze into 6 groups: Oxidoreductases, transferases, hydrolases, lyases, isomearses, ligases.

Oxidoreductases - Oxidoreductase are the enzymes that catalyze oxidation-reduction reactions. These enzymes are important as these reactions are responsible for the production of heat and energy.

Transferases - Transferases are the enzymes that catalyze reactions where transfer of functional group between two substrates takes place.

Hydrolases - Hydrolases are also known as hydrolytic enzymes, they catalyze the hydrolysis reactions of carbohydrates, proteins and esters.

Lyases - Lyases are enzymes that catalyze the reaction involving the removal of groups from substrates by processes other than hydrolysis by the formation of double bonds.

Isomerases - Isomerases are enzymes that catalyze the reactions where interconversion of cis-trans isomers is involved.

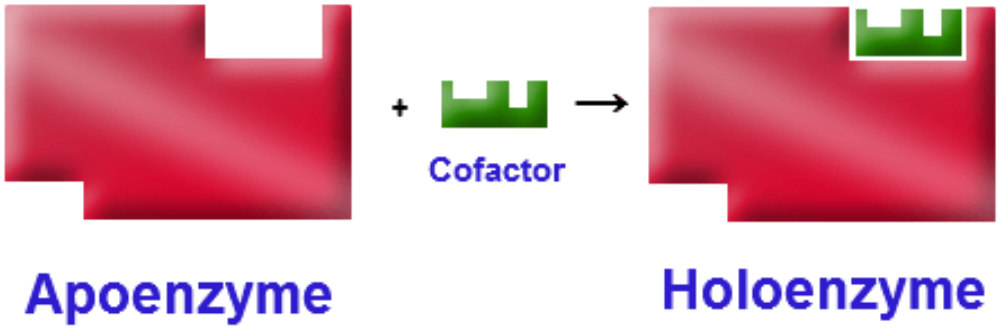
Ligases - Ligases are also known as synthases, these are the enzymes that catalyze the reactions where coupling of two compounds is involved with the breaking of pyrophosphate bonds.

Structure of enzymes

Enzymes are proteins, like the proteins the enzymes contain chains of amino acids linked together. The characteristic of an enzyme is determined by the sequence of amino acid arrangement. When the bonds between the amino acid are weak, they may be broken by conditions of high temperatures or high levels of acids. When these bonds are broken, the enzymes become nonfunctional. The enzymes that take part in the chemical reaction do not undergo permanent changes and hence they remain unchanged to the end of the reaction.

Enzymes are highly selective, they catalyze specific reactions only. Enzymes have a part of a molecule where it just has the shape where only certain kind of substrate can bind to it, this site of activity is known as the *'active site'*. The molecules that react and bind to the enzyme is known as the *'substrate'*.

Most of the enzymes consists of the protein and the non protein part called the '*cofactor'*. The proteins in the enzymes are usually globular proteins. The protein part of the enzymes are known '*apoenzyme',* while the non-protein part is known as the cofactor. Together the apoenzyme and cofactors are known as the '*holoenzyme'*.



Cofactors may be of three types: prosthetic groups, activators and coenzymes.

Prosthetic groups are organic groups that are permanently bound to the enzyme. Example: Heme groups of cytochromes and bitotin group of acetyl-CoA carboxylase.

Activators are cations- they are positively charged metal ions. Example: Fe - cytochrome oxidase, CU - catalase, Zn - alcohol dehydrogenase, Mg - glucose - 6 - phosphate, etc.

Coenzymes are organic molecules, usually vitamins or made from vitamins. they are not bound permanently to the enzyme, but they combine with the enzyme-substrate complex temporarily. Example: FAD - Flavin Adenine Dinucleotide, FMN - Flavin Mono Nucleotide, NAD - Nicotinamide Adenine Dinucleotide, NADP - Nicotinamide Adenine Dinucleotide.

Function of Enzymes

Biological Functions of Enzymes:

- Enzymes perform a wide variety of functions in living organisms.
- They are major components in signal transduction and cell regulation, kinases and phosphatases help in this function.

- They take part in movement with the help of the protein myosin which aids in muscle contraction.
- Also other ATPases in the cell membrane acts as ion pumps in active transport mechanism.
- Enzymes present in the viruses are for infecting cell.
- Enzymes play a important role in the digestive activity of the enzymes.
- Amylases and proteases are enzyme that breakdown large molecules into absorbable molecules.
- Variousa enzymes work together in a order forming metabolic pathways. Example: Glycolysis.

Uses of microbial enzymes

Enzymes are being used in many areas for production of useful products. In the textile industry lipases are used for the removal of size lubricants, which increases fabrics absorbance ability for improved levelness in dyeing. The hydrolytic lipases are commercially very important, and their addition to detergents is mainly used in laundries and household dishwashers. On the other side, to modify the food flavour by synthesis of esters of short-chain fatty acids and alcohols lipases have been frequently used. Lipases play a major role in the fermentative steps during manufacturing of sausage and also to measure changes in long-chain fatty acid liberated during ripening. Previously, lipases of different microbial sources were used for refining rice flavour, modifying soybean milk, and for enhancing the aroma and speed up the fermentation of apple wine.

Enzymes with Special Characteristics

Special characteristics of microbial enzymes include their capability and appreciable activity under abnormal conditions, mainly of temperature and pH. Hence, certain microbial enzymes are categorized as thermophilic, acidophilic or alkalophilic. Microorganisms with systems of thermostable enzymes that can

function at higher than normal reaction temperatures would decrease the possibility of microbial contamination in large scale industrial reactions of prolonged durations. The quality of thermostability in enzymes promotes the breakdown and digestion of raw materials; also the higher reaction temperature enhances the penetration of enzymes. Thermophilic xylanase are considered to be of 59 commercial interest in many industries particularly in the mashing process of brewing. The thermostable plant xerophytic isoforms of laccase enzyme are considered to be useful for their applications in textile, dyeing, pulping and bioremediation .

Types of Enzymes

A number of different enzymes exist with various functions. Microbial proteases are hydrolytic enzymes that have been extensively studied. Proteases prepared from microbial systems are of three types: acidic, neutral and alkaline. Alkaline proteases are efficient under alkaline pH conditions and consist of a serine residue at their active site. Alkaline proteases have shown their capability to work under high pH, temperature and in presence of inhibitory compounds. Insoluble and fibrous proteins consisting of feathers and wool are considered as keratinases. The protein is abundantly available as a by-product from keratinous wastes, representing a valuable source of proteins and amino acids that could be useful for animal feeds or as a source of nitrogen for plants .

Many significant enzymes like amylases can be used in industry for starch conversion. Amylolytic enzymes act on starch and related oligo and polysaccharides. The baking industry uses amylases to delay the staling of bread and other baked products; the paper industry uses amylases for the reduction of starch viscosity to achieve the appropriate coating of paper. Amylase enzyme is used in the textile industry for warp sizing of textile fibers, and used as a digestive aid in the pharmaceutical industry. Another class of enzymes named as xylanases have established their uses in the food, pulp, paper and textile industries, agri-industrial residues utilization, and ethanol and animal feed production.

Isolation of microbial enzymes

The industrial enzymes are drived from plants, animals, and microorganisms. However, the microorganisms are in use to produce these enzymes owing to an important yield obtained from them, and reduction of cost and labor. Most of the industrial enzymes are secreted by *Bacillus* and *Aspergillus* species. Some industrial enzymes having same application can be co-produced in a single fermentation medium. In this case, the production process becomes cost effective and the enzyme stability is assured. The role of medium composition optimization is to maintain the balance between the different medium ingredients thereby preventing the amount of unused components at the end of fermentation process. Each bacterial or fungal species has its own growth conditions to produce industrial enzymes in a significant amount. Statistical methods are also employed to produce the industrial enzymes in adequate amounts. The production of microbial industrial enzymes under optimized conditions to get enzymes with desirable properties is a continuous exercise.

Isolation and screening of industrial enzyme-producing microorganism

The industrial enzyme-producing microorganisms are generally isolated from soil samples collected from various places. They are also obtained from plant bark , watery environment, skim milk, marine sediment, municipal solid wastes and from grapes. The microorganisms producing industrial enzymes are usually screened from samples by serial dilution followed by spread plating on specific agar medium depending on the enzyme of interest to be produced by microorganisms . Therefore, the selected isolation and screening medium contains an industrial enzyme inducer. For instance, the laccase production by ***Scytalidium lignicola*** was stimulated by CuSO4 and MgSO4. Some proteases may require casein, skim milk or gelatin as inducer. The amylases, cellulases, and lipases may require starches, carboxymethylcellulose and oil substances as inducers , respectively.

The formation of clear zone of specific substrate hydrolysis on agar medium plates around the microorganism is an indication of an industrial enzyme secretion by the microorganism. For example, the industrial microbial amylases produce clear zones after starch hydrolysis. Similarly, a clear zone of casein/gelatin hydrolysis on agar plates around the organism is observed in the case of protease. Furthermore, the tributyrin / Tween 80 hydrolysis or color change from red to yellow for oilphenol red agar plate or appearance of orange fluorescent halos around industrial microorganism under UV light at 350 nm, is seen for industrial lipase secreted by microorganisms. The color change is due to the release of fatty acids from oil hydrolysis that lowers the medium to the acidic pH (yellow color). The appearance of orange fluorescent halos occurred as a result of the complexation between the Rhodamine B and the oil hydrolysis products (fatty acids, mono- and diglycerides).

Isolation of Bacteria with Potential of Producing Extracellular Enzymes (Amylase, Cellulase and Protease) from Soil Samples

1. Material and methods

1.1 Sample Collection, Isolation and Identification of Bacteria Soil samples deposited with waste materials were collected from eight different locations .The samples were diluted and appropriate dilution was inoculated into Nutrient agar using pour plate technique. They were incubated at 37°C for 24 to 48 hours. Distinct colonies from plates were streaked out on Nutrient agar until pure cultures were obtained. Isolated bacteria were stored in Nutrient agar slant for further use. The isolates were identified using their morphological and biochemical characteristics with reference to Bergey Manual of Systematic Bacteriology.

2.1 Extracellular Enzymes Screening

Amylolytic property of the isolated bacteria was screened using Nutrient agar supplemented with 1% starch. Clear zone around the organism after flooding with

Gram's iodine indicates the ability of the bacterium to produce amylase. Nutrient agar supplemented with 1% carboxy methyl cellulose (CMC) was used to screen ability of the isolated bacteria for cellulase production. Appearance of a clear zone against red colour of Congo red shows that the organism has ability to produce cellulose. Proteolytic ability of isolates were screened using nutrient agar supplemented with 1% skimmed milk. A clear halo along the line of streak indicates ability of the bacterium to produce protease. Relative activities of enzymes were determined using the method of Adesina and Onilude.

RESULTS

An amylase-producing bacterium is shown in Plate 1A. There was a clear zone around the line of streak of the bacterium which was due to its ability to hydrolyse starch. Plate 1B shows a bacterium with the ability to produce cellulase. The bacterium hydrolysed Carboxy Methyl Cellulose (CMC) agar around the line of streak as indicated by clear zone. A protease-producing bacterium is shown in Plate 1C. There was a clear halo along the line of streak which is as a result of the ability of the bacterium to hydrolyse skimmed milk agar. The most frequent of all the bacteria isolated from soil samples in this study was *Bacillus* species.

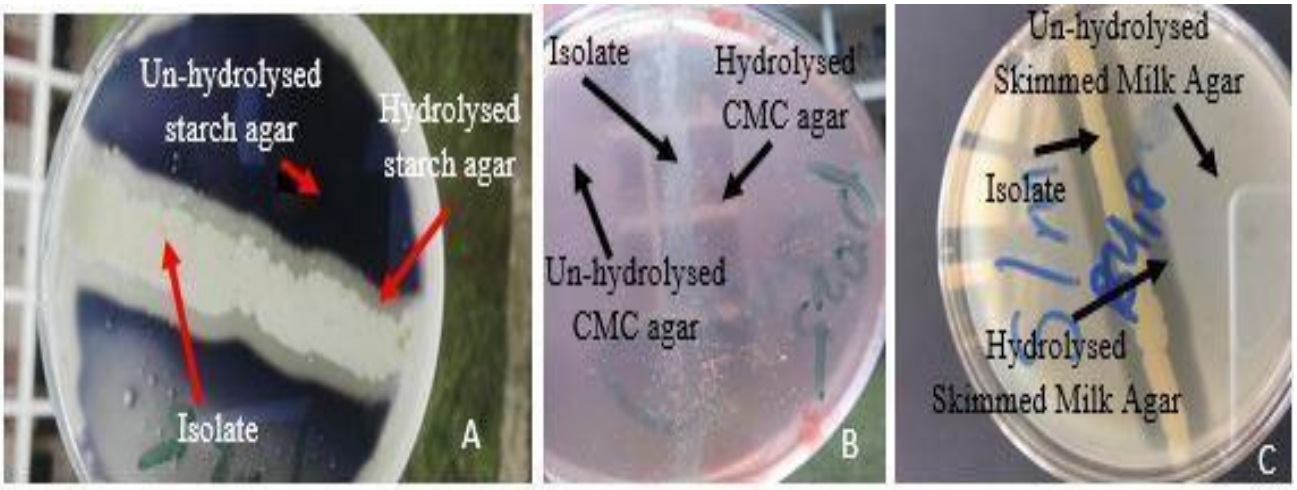


Plate 1A. Amylase-producing bacterium on starch agar plate Plate 1B. Cellulase-producing bacterium on Carboxy Methyl Cellulose (CMC) agar platePlate 1C. Protease-producing bacterium on skimmed milk agar plate

Materials and Method:

Sample Requirement:

• Soil Sample from

I. Soil sample collected

II. Soil sample collected from a Cattle Farm situated in india.

Materials:

• Glass rod • Test tubes • Petri plates • Conical flask • Incubator, HEPA Filter & Autoclave

Chemicals:

Isolation of Laccase producers:

• Basic medium containing 0.5% ammonium tartarate, 0.1% malt extract, 0.001%, calcium chloride, 0.1% sodium chloride, 0.001% ferric chloride, 0.1% lignin.

• Nutrient agar containing (0.02%) catechin.

• Sodium phosphate buffer pH 6.0 (100 mM)

• ml of guaiacol (10 mM)

Isolation of Cellulase producers:

• 1.0%peptone,

• 1.0% carboxymethylcellulose(CMC),

• 0.2 % K2HPO4, 1 % agar, 0.03 %

• MgSO4.7H2O,

• 0.25 % (NH4)2SO4

• 0.2% gelatine.

• pH of medium pH 7

• 1 % Congo red

• 1M NaCl

Isolation of Amylase producers:

• NaCl

• Yeast extract

• Beef extract

• Starch

• Agar

• pH of medium 7.4+-0.2

• Gram's Iodine solution

Isolation of alkaline protease producing bacteria

• Skimmed milk agar plates (g/l) (skim milk powder-28, casein enzymic hydrolysate-5, yeast extract-2.5, dextrose-1, NaCl-0.5, agar-15) with pH 9.0 (using phosphate buffer)

Procedure:

Isolation procedure of Laccase producer:

The methodology given by V. Bhuvaneshwari et al.(2015) on the isolation, optimization and production of laccase enzyme from soli bacteria, was followed to isolate strains of bacteria capable of producing Laccase.

Enrichment and isolation of microorganisms:

One gram of the soil sample was diluted by transferring the sample into 100 mL of sterile distilled water. About 1 mL of diluted sample was transferred to 20mL of basic medium containing 0.5% ammonium tartarate, 0.1% malt extract, 0.001% calcium chloride, 0.1% sodium chloride, 0.001% ferric chloride, 0.1% lignin, incubated in shaker incubator at 37 °C for 3 days. The lignin was prepared from peanut hull. The enriched samples were then serially diluted by transferring into test tubes so that sample concentrations were obtained from 10-1 to10-10. About 0.1 mL aliquots of 10-2 to 10-10 were pipetted out into sterilized nutrient agar plates

containing (0.02%) catechin and spread plated. The plates were then incubated at 37 °C. The coloured zone formed around the colony indicates the production of laccase.

Screening of Laccase producing microbes:

The morphologically different strains which produced colour in the nutrient agar plates by reducing the Catechin were then streaked on nutrient agar slants in test tubes and incubated for 2-3 days.

Growth of isolated laccase producing microorganisms:

The morphologically different colonies were isolated and screened for laccase activity. Each isolated colony was cultured on basic medium (Nutrient Broth) supplemented with 0.5% glucose (production medium) at 37 °C for 24h in orbital shaker. The inoculated broth was left in the shaker at 37 °C for 24 h.

Laccase enzyme assay:

(a) Enzyme production: Following incubation of the production medium for appropriate period, the contents in the flask were centrifuged at 8000 rpm for 5 min and the supernatant was collected which is an extracellular enzyme.

(b) Enzyme assay: Laccase activity was determined using catechin as the substrateaccording to the method of Sandhu and Arora [1985]. The assay mixture contained 4.80 ml of sodium phosphate buffer pH 6.0 (100 mM), 0.1 ml of catechin (10 mM) and 0.1 ml of enzyme extract. The kinetic reaction was spectrophotometrically recorded at 470 nm (ε = 21,600/M/cm) incubated at 60 °C for 30 min, in an UV-Visible spectrophotometer. The blank contained all the assay constituents except the active enzyme, buffer or heat inactivated enzyme was used in its place. The enzyme activity was expressed as U/l. One unit of enzyme was defined as the amount of enzyme that liberated 1µM of product/minute/mL.

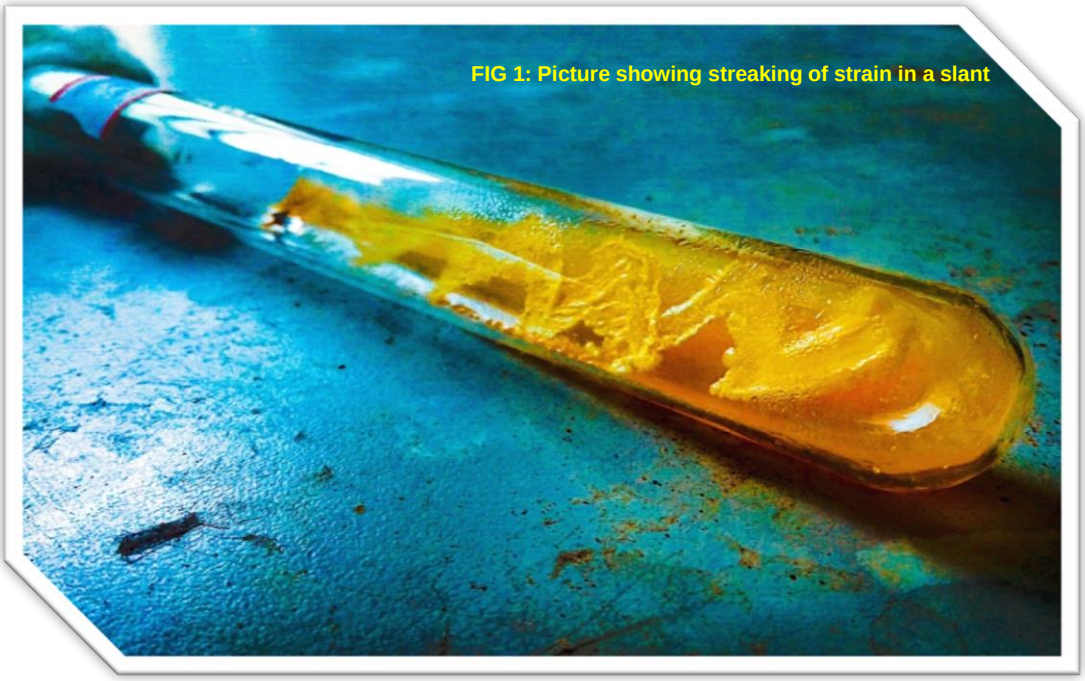


FIG 1: Picture showing streaking of strain in a slant

Results: From the first procedure of isolating laccase producers we could isolate four strains based on their colony morphology and colour. We depicted each of the strains by naming them; i. Sample I, ii. Sample II, iii. Sample III and Sample IV (*shown in FIG 2*). All these strains were laccase producers.

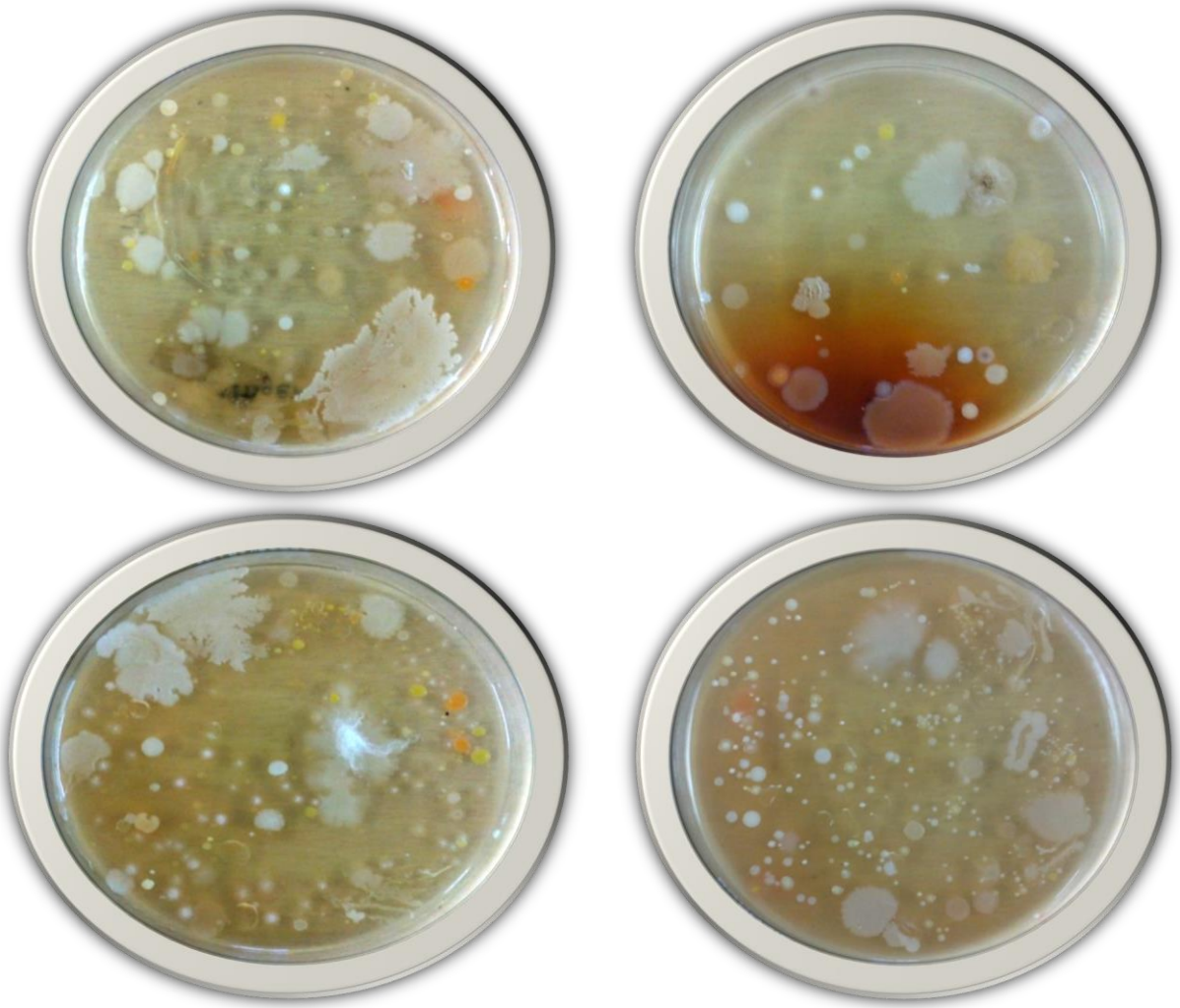


FIG 2: SAMPLES I, II, III & IV

Isolation procedure of Cellulose producer strains*:*

The methodology illustrated by *Basavaraj et al.* (2004) and of *Shanmugapriya et al.* (2012), were chosen as the standard procedures for the isolation of strains producing cellulase.

Isolation of microorganisms:

The collected soil sample was serially diluted into sterilized test tubes and was incubated for growth at 37^0C. The incubated CMC agar plates were flooded with 1 % Congo red and allowed to stand for 15 min at room temperature. 1M NaCl was thoroughly used for counterstaining the plates. Clear zones were appeared around growing bacterial colonies indicating cellulose hydrolysis. The bacterial colonies having clear zone were selected for identification and cellulase production. Further bacterial strains were purified by repeated streaking. The purified colonies were preserved at 4 ^{0}C.

The selected bacterial cultures were individually maintained on CMC agar plates at 4 ^{0}C. The selected bacterial cultures were inoculated in broth medium containing 0.03 % MgSO4, 0.2 % K2HPO4, 1 % glucose, 0.25 % (NH4)2SO4 and 1 % peptone at pH 7 for 24 Hrs of incubation period. After the incubation period these bacterial cells were used as inoculum.

Screening of cellulose producing microbes :

The isolated Bacterial strains were screened for cellulase enzyme production in submerged fermentation process. Fermentation medium was prepared using 0.2 % K2HPO4, 0.03 % MgSO4, 1 % peptone, 0.25 % (NH4)2SO4 and autoclaved at 121oC for 15min. After autoclave, the medium was inoculated with 1 ml of bacterial isolates and incubated in a rotary shaker at 35 C for 24 hrs of fermentation period with agitation speed of 140 rpm. After fermentation, the broth was centrifuged at 14000 × g for 10 min at 4 C. The supernatant obtained after centrifugation served as crude enzyme source.

Results: After this we tried to find whether these strains were capable of performing other enzymatic activities or not. So we performed some other tests to find if they can perform some other enzymatic activities. Firstly we did cellulose isolation and it was found that sample I gave prominent positive result and sample III gave sparing positive result. Sample IV and II on the other hand gave negative response (as shown in FIG 3).

FIG 2: SAMPLES I, III & IV

Isolation of strains producing Amylase: The procedure demonstrated by *Gebreselema Gebreyohannes* (2015), on isolation and optimization of amylase producing bacteria from soil sample**,** were applied to perform spot test to isolate the strains of bacteria capable of producing Amylase.

Isolation of microorganisms:

Isolation of soil bacteria was performed by serial dilution and spread plate method. One gram of soil sample was serially diluted in sterilized distilled water to get a concentration range from 10- 1 to 10-6. A volume of 0.1 ml of each dilution was transferred aseptically to starch agar plates. The sample was spread uniformly. The plates were incubated at 37°C for 24 hr. The bacterial isolates were further sub cultured to obtain pure culture. Pure isolates on starch agar slants were maintained at 4ºC.

Screening of cellulose producing microbes

Bacterial isolates were screened for amylolytic activity by starch hydrolysis test on starch agar plate. The microbial isolates were streaked on the starch agar plate and incubated at 37°C for 48 hours. After incubation iodine solution was flooded with dropper for 30 seconds on the starch agar plate. Presence of blue colour around the growth indicates negative result and a clear zone of hydrolysis around the growth indicates positive result. The isolates produced clear zones of hydrolysis were considered as amylase producers and were further investigated.

Results:

Next we tried to find if they were amylase producers also. Experiments showed that sample III fetched positive while all the remaining strains gave negative results (*refer FIG 4*).

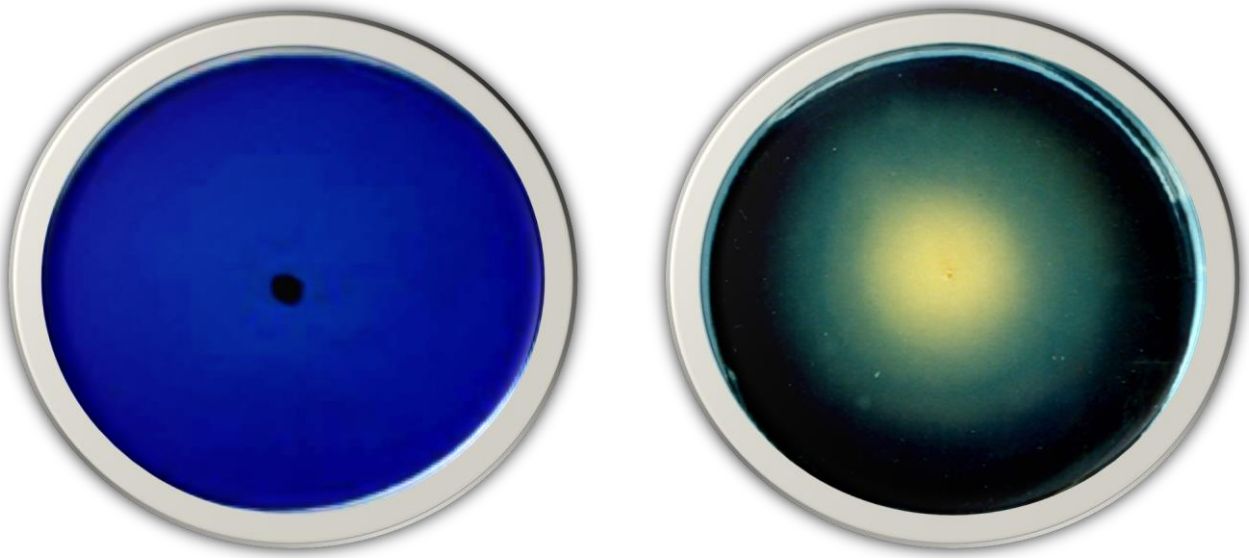


FIG 4: ***SAMPLES I & III***

Isolation of strains producing protease:

The spot test illustrated by Narendra et al (2012), for identification of protease producing bacteria from soil was applied to check for the protease production capabilities.

Each sample was serially diluted and spread plated on Skimmed milk agar plates (g/l) (skim milk powder-28, casein enzymic hydrolysate-5, yeast extract-2.5, dextrose-1, NaCl-0.5, agar-15) with pH 9.0 (using phosphate buffer) and incubated at 37°C for 48 hrs. The formation of clear zone around the colonies confirms the production of alkaline protease. The colonies that had formed a clear zone around the growth were considered as protease positive isolates.

Results: Our last experiment was to check if they were protease producers also. Sample IV showed positive response this time and others gave negative responses (*FIG 5*).

Figure (5): I, IV

Isolation of Fungal Enzymes (Amylase, Cellulase and Protease) from Tropical Rainforest in Mexico

Extracellular enzymes of fungal origin, both oxidoreductive and hydrolytic, have been reported for various industrial and biotechnological applications, like medicine, agriculture, pulp and paper, textiles , detergents , food processing and biofuels industries ; as well as bioremediation. In addition, fungal enzymes have a more significant advantage over those derived from plants or animals due to their easy handling, rapid production in low-cost media, higher yields, and catalytic activity.

1.Materials and Methods

1.1. Biological Sampling

The samples were collected from three conserved tropical sites in the sierra region of the south of Mexico, the annual average temperature is between 22 and 26.8 ◦C.

2.1 Isolation of the Fungi

The fungi were isolated in potato dextrose agar (PDA) with chloramphenicol (15 µg/mL) to reduce bacterial growth. Soil fungi were isolated by washing and filtrating particles techniques; after removing the moisture excess, five soil particles

were inoculated on each plate. The fungal strains obtained from leaf litter and sporocarps on decaying wood were superficially washed with 2% sodium hypochlorite for 1 min, 70% ethanol for 1 min, and three washes were performed with sterile distilled water. The sporocarps (MAF) were cut into small fragments and inoculated with a fine needle, while the leaf litter was cut into fragments of 1 cm2 , and five fragments were inoculated in the plate. The plates were incubated at 28 ◦C, and periodic checks were carried out every third day for one month. Emerging fungi were transferred onto new plates with PDA until obtaining pure cultures.

3.1. Morphological Identification

1250 macroscopic (MAF) and microscopic fungal (MIF) were isolated from soil, leaf litter, and wood. The strains were grouped by source, morphotype, and percentage of occurrence (data not shown); later, 50 strains from each source were selected (soil, leaf litter, and wood), excluding the typical morphologies of *Penicillium*, *Cladosporium*, and *Trichoderma*. The fungal isolates were identified according to macroscopic and microscopic characteristics—such as mycelia, fruiting bodies, arrangement of conidia, among others— using taxonomic keys.

The MIF isolates from leaf litter and soil that did not show spores were inoculated in corn agar, oatmeal agar, potato carrot agar, V8 agar, humic acid agar, and leaf litter agar to promote sporulation, incubating them for six weeks under continuous black light at 28 ◦C. All isolates were maintained in PDA slant tubes and stored in 20% glycerol at −80 ◦C, and mycelium plugs were stored in sterile distilled water at room temperature (28–30 ◦C).

Screening of fungal enzymes

For the enzyme screening of the 150 selected strains, plates with minimal mineral medium (0.6 g/L NH_4NO_3, 0.2 g/L KH_2PO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.001 g/L $FeSO_4 \cdot 7H_2O$, 15 g/L bacteriological agar) were supplemented with substrates specific: 0.5 mM 2,20 -azinobis 3-ethylbenthiazoline-6-sulfonic acid (ABTS)

and 0.9 mM guaiacol for laccases, 6% (v/v) olive oil, 0.2% (v/v) Tween 80, and 0.001% rhodamine B solution, at pH 7.0 for lipases , 1% soluble starch, at pH 6.0 for amylases, 1% birch xylan for xylanases , and 1% skim milk powder for proteases.

All the media were sterilized for 15 min at 120 ◦C. An inoculum of the fungus (0.5 cm2) was placed in the center of the Petri dishes and incubated at 28 ◦C for 15 days. All strains that showed **a blue-green oxidation halo for** laccases were considered positive, as well as **a clear area around the fungal growth product of the hydrolysis of starch**, xylan, and casein, **while an orange fluorescence halo was observed for lipase activity**. All the tests were carried out in triplicate. The activity was reported as potency index (PI) measured every 24 h, as the halo diameter formed between the mycelial growth's diameter.

Basal Medium for Enzyme Production

To evaluate laccase activity, the modified medium], was used (10 g/L fructose, 2.5 g/L malt extract, 2.5 g/L yeast extract, 1 g/L KH2PO4, 0.05 g/L (NH4)2SO4, 0.5 g/L MgSO4, 0.01 g/L CaCl2, 0.001 g/L MnSO4, 0.001 g/L ZnSO4, and 0.2 mM CuSO4·5H2O, adjusted at pH 4.8). For amylase and xylanase activity, we used 2.5 g/L yeast extract, 0.6 g/L NH4NO3, 0.2 g/L KH2PO4, 0.2 g/L MgSO4·7H2O, 0.001 g/L FeSO4·7H2O supplement 10 g/L **soluble starch** for amylase and 10 g/L **birch xylan, 5 g/L wheat bran for xylanase**, both media were adjusted to pH 6.0.

To **evaluate protease and lipase activity the following medium was used**: 2.0 g/L yeast extract, 0.02 g/L MgSO4, 2.0 g/L glucose, and 0.1 g/L KH2PO4, supplemented with 20 g/L casein and 2% v/v olive oil, respectively.

Enzyme Production by Submerged Fermentation

The submerged fermentation medium for enzyme production was sterilized for 20 min at 120 ◦C; 100 mL of the sterile basal medium was prepared as earlier described with the appropriate carbon source in 250 mL flasks. Each fungus was

grown from two to seven days on PDA, and 8 mm agar plugs, then were cut from the agar and transferred in flasks, keeping them in an orbital shaking at 140 rpm at 28 ◦C for 16 days.

The enzyme quantification was performed by protein content according to the Lowry method. For this purpose, 5 mL of the supernatant were taken every second day, centrifuging at 1487× g for 10 min, followed by filtration on Whatman filter paper number 1. The experiments were performed in triplicate for each fungal strain.

Statistical Analysis All enzyme quantification was performed in triplicate, and results were analyzed with multiple comparisons of means, performing a one-way analysis of variance (ANOVA), followed by a Tukey test. A significance of $\alpha = 0.05$ was considered in the analyses with the statistical package Stagraphics.

Results:

Qualitative Plate Test

The laccase activity was detected by the oxidation of the ABTS The nonphenolic dye (blue-green halo) and guaiacol (brown halo) around the colony, mainly of fungi known as lignin degraders or white rot. ble 1). **Protease activity** was evident with a clear zone (substrate hydrolyzed) around the colony

Regarding the detection of amylase, positive results were visualized after adding an iodine solution (0.3% iodine and 0.6% potassium iodide) that caused a clear zone around the colonies because of the hydrolysis of starch. **In xylanases,** the visualization was by adding a Congo red solution (0.4%) that stains the plates red, observing a lighter area around the colonies that indicate the presence of xylanase activity. **Regarding lipase activity**, the positive strains were determined by forming an orange- fluorescent halo around the colonies visible under UV light at 350 nm, a product of the interaction of rhodamine B with the fatty acids released during the enzymatic hydrolysis of the triacylglycerols.

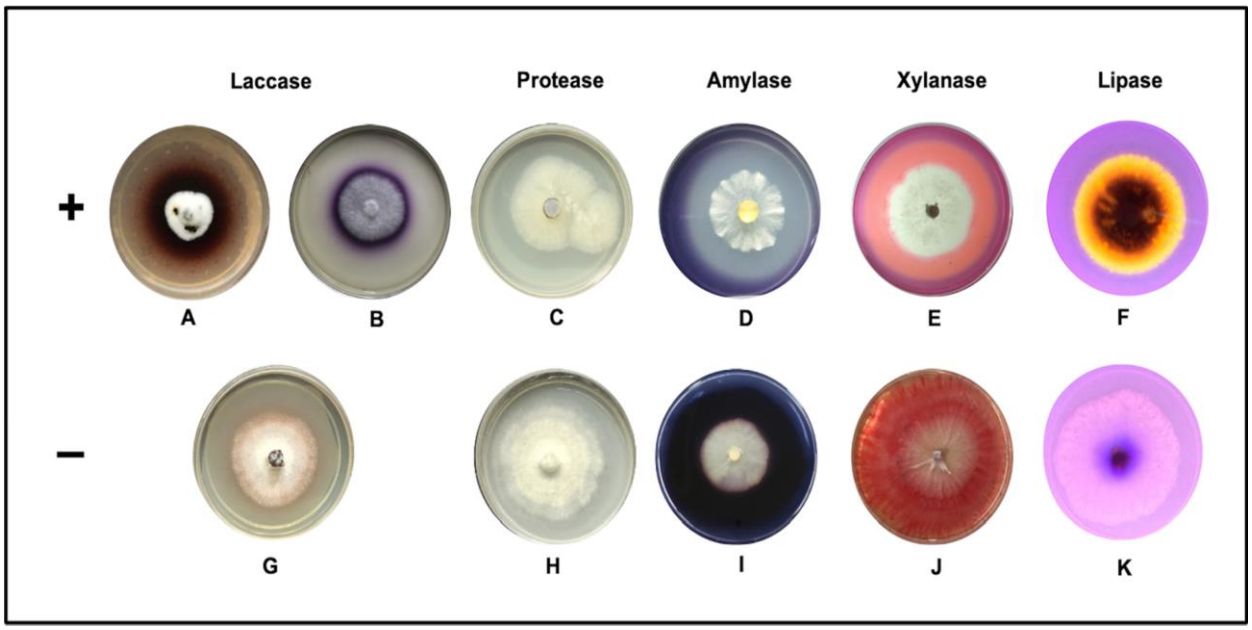


Figure 2. Qualitative enzymatic assay on solid medium with visible enzymatic activity (**A**–**F**) and without activity (**G**–**K**).

Isolation and Screening of Extracellular Protease Enzyme from Fungal Isolates of Soil

Sample Collection

The soil samples were collected from the surface of the soil and at a depth of about 15 cm and taken with the help of a sterilized hand trowel was used to collect about 100 g each of the top soil and depths according to Oyeleke et al., (2010).

Isolation and purification of fungi

The serial dilution technique are used for the isolation of fungi from the soil. The twenty-five grams of the soil samples were mixed with a solution 225 ml of 1% peptone water with some modification. Five screw cap test tubes with nine ml distilled water were autoclaved and arranged into the laminar flow hood for further processing, and serial dilutions were made from 10-1, 10-3, and 10-5. First, third and fifth dilution were used to obtain fungal colonies.

Then one milliliter (1ml) of soil suspension from the third dilution was taken and spread on the Potato Dextrose Agar (PDA) media plates using a glass rod spreader, sterilized by dipping in 70 % ethanol and flaming. The plates were incubated at room temperature (27°C) for 5 days, after spreading.

A mixture of various soil-borne fungi was grown on PDA medium, and a single spore of each fungus transferred to new PDA medium plate by a sterilized needle for isolation of pure culture. After ensuring purity, the cultures were subcultured on PDA plates and allowed to grow for a period of 5-7 days and subsequently stored at 4ºC as stock cultures.

Potato dextrose agar (PDA) preparation Suspend 39 g of the Potato Dextrose Agar (PDA) medium (Oxoid, England) in 1000 ml of distilled water. Autoclaved at 121°C for 15 minutes then poured into plates. The plates were sterilized in fume hood cabinet.

Preparation of Skim Milk Agar (SMA)

Skim milk agar medium (Oxoid, England) is used for primary screening of protease producing fungi by mixing 25 g of nonfat dry milk was mixed with 250 ml of distilled water. The mixture was stirred thoroughly and autoclaved at 121°C for 15 min. The pH of the medium adjusted by using the pH meter (OMEGA, England) maintained at pH 6, the suspension of 2.5% agar (Oxoid, England) used for solidification were autoclaved at 121°C for 15 min. For plating, skim milk and agar solutions were held in a water bath at 50°C and then the skim milk was poured into the agar flask and mixed thoroughly. To restrict the bacterial growth 1 mg/1000 ml ampicillin (Duchefa Brochemie B.A, Netherlands) was added to the media. The skim milk agar was poured quickly into plates then kept at 4°C until used.

Screening for protease production

Skim milk agar medium is used for screening of protease production by fungi. The pure culture isolates were streak on the skim milk agar plates and incubated at room temperature (27°C) for four days. Then the appearance of clear zone in the medium around the colony indicates protease activity. The zones diameter were

measured in mm and results are recorded. The enzymatic index (EI) expressed as R/r, which R is the degradation zone diameter and r is the colony diameter. The species that exhibits maximum clear zone selected for further identification.

Identification of isolated fungi

The isolated fungi were subcultured on PDA and allowed to grow and sporulation. Then from their colony and morphological characteristics, the fungi were identified. The slides observed under the microscope then fungi were identified by following the mycological literature. The following morphological characteristics evaluated the colony growth (length, width, presence or absence of aerial mycelium, colony color, a presence of wrinkles and furrows, and pigment production) (Tarman et al., 2011).

Results:

The fungal isolates were screened for their ability to produce protease enzymes. Majority of the isolates showed protease production activity as shown in Table 3. The maximum production observed after 96 hours. Based on the skim milk agar experiment for protease detection, total of eight isolates from the soil at 15 cm depth exhibited protease production activity while seven isolates from the surface produced high protease enzymes. At 48 hours 14L3S isolate zone was 12.75 mm and increased to 15.75 mm after 72 hrs, then reached to 17 mm after 96 hrs. 17L1D did not show any protease production activity at 48 hrs, while the protease production started after 72 hrs to finally reach 3.25 mm after 96 hrs.

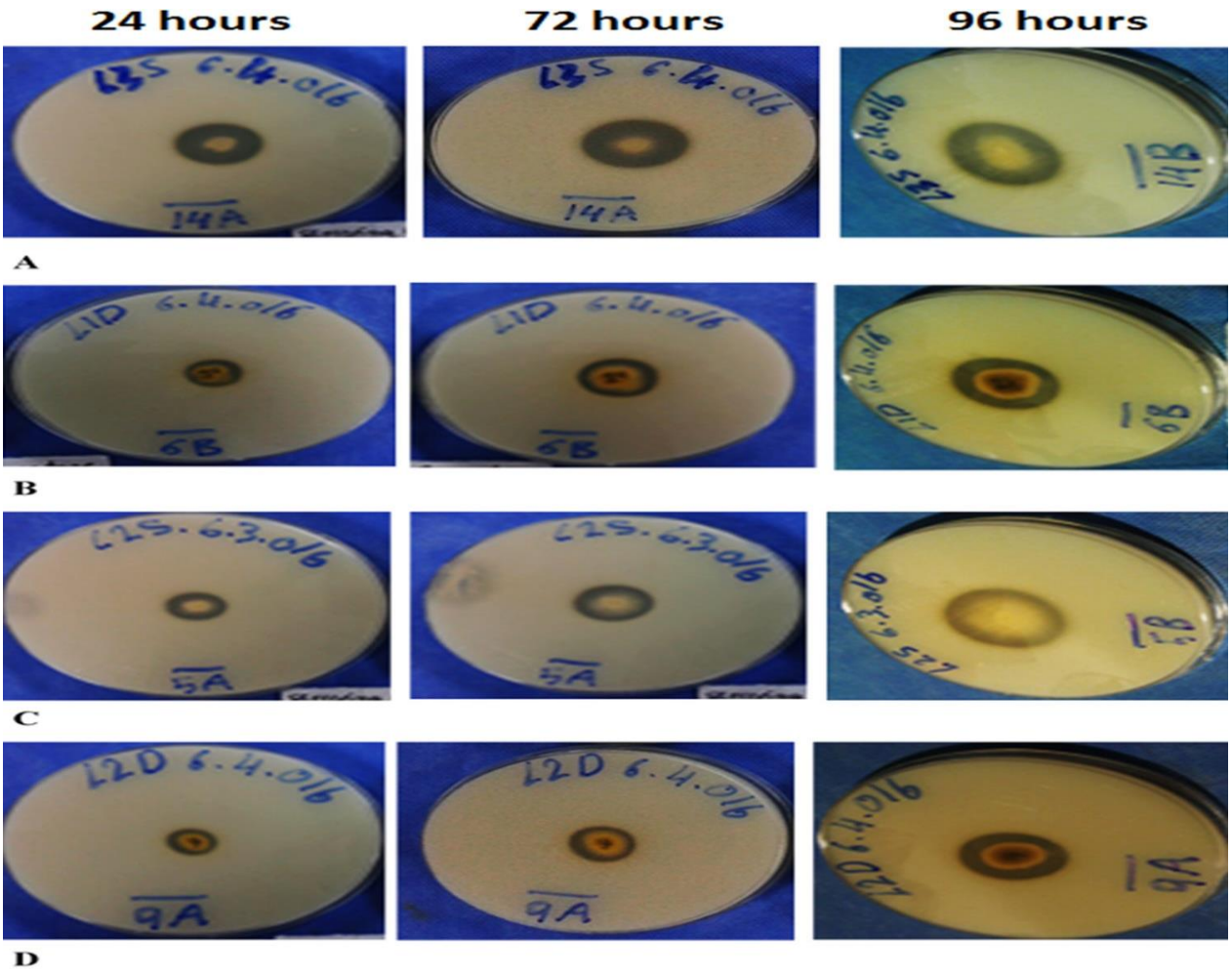


Fig. 1. Clearance Zone diameter around the fungal isolates A) 14L3S, B) 6L1D, C) 5L2S, and D) 9L2D.

Isolation and characterization of amylase enzyme from selected fungal strains

Sample collection

Soil samples were collected in a sterile polyethylene container at the depth of 15 cm pH 4.8 temperatures 25° C which was measured at the site of sampling area by using centimeter, pH meter and thermometer respectively. To restrict the bacterial growth 1 mg/1000 ml ampicillin (Duchefa Brochemie B.A, Netherlands) was added to the media. The skim milk agar was poured quickly into plates then kept at 4°C until used.

Isolation of fungi from soil sample

Fungal colonies were isolated by diluting 10 gm of soil samples in 100 ml of sterile distilled water by serial dilution method. Potato dextrose agar (PDA) (Potato 200 gm, Dextrose 20 gm, Agar 20 gm, Distilled water 1000 ml) media was prepared, autoclaved and poured in sterile Petri plates and 0.1 ml of soil samples were diluted up to 10-5 dilutions and spread on respective solidified PDA plates. The inoculated plates were incubated at ambient temperature for 120 hours (5 days).

Fungal isolates were differentiated on the basis of their physical characteristics such as hyphae and mycelium, which was obtained after incubation. Successive purification was carried out on sterile PDA plates by point inoculation and incubated by following the above method until pure isolate was obtained. Pure isolates were stored at 4° C using slants for further work.

Screening of fungal isolates for amylase production

To select the best amylase producing strains 108 fungal isolates were grown in starch agar media comprising the following, in gmL- 1; yeast extract 1.5, peptone 0.5, sodium chloride 1.5, starch 10, agar 15, and make the media of the pH similar with pH of soil sample. The isolates were inoculated on sterile solidified starch agar plates a blank without inoculation was also maintained for comparison. Then the plates were incubated at room temperature for 120 hours after that all the plates along with blank was flooded with iodine and observed for zone of hydrolysis.

Inoculums preparation

Inoculums were prepared by adding 10 ml of sterilized distilled water into a sporulated 5 days old PDA slant culture. An inoculums needle was used to displace the spore clusters under sterilized conditions and then it was shaken thoroughly to prepare homogenized spore suspension.

Production of crude amylase using solid state fermentation Solid state Fermentation (SSF) medium was used for enzyme production from selected isolates using wheat bran as a sole carbon source supplemented with the following nutrients g/100 ml (%): glucose, 0.05; NH4 SO4 , 0.02; trisodium citrate, 0.027; MgSO4 , 0.008; KH2 PO4 , 0.19 and 10g of wheat bran was moistened with above nutrient solution to a level of (v/w) 65% in 250 ml size Erlenmeyer flasks was used for cultivation of the fungal isolates. The moisture level of the wheat bran was removed after overnight oven drying at 105° C. To extract the released enzyme the moldy bran was soaked in 10 mM acetate buffer pH 4.8 in (w/v) 1:10 ratio and shacked for 1hr at 120 rpm on the shaker, then the crude enzyme from SSF medium was harvested by centrifugation repeatedly at 5,000 rpm for 10 min.

Effect of pH on activity and stability of amylase: To determine the pH activity of the fungal amylases the crude enzyme was assayed using 1% soluble starch prepared in an acetate buffer systems pH 2.0 to 11.0. The activity of enzyme was determined by using the standard assay conditions. To determine the effect of pH on the activity of the glucoamylase enzyme incubated in a pH range 2.0 to 11.0 (at one interval). In this study as the pH value increase amylase activity also increases and its highest activity was observed at pH 5.0. The maximum amylase activity was found at pH 5.

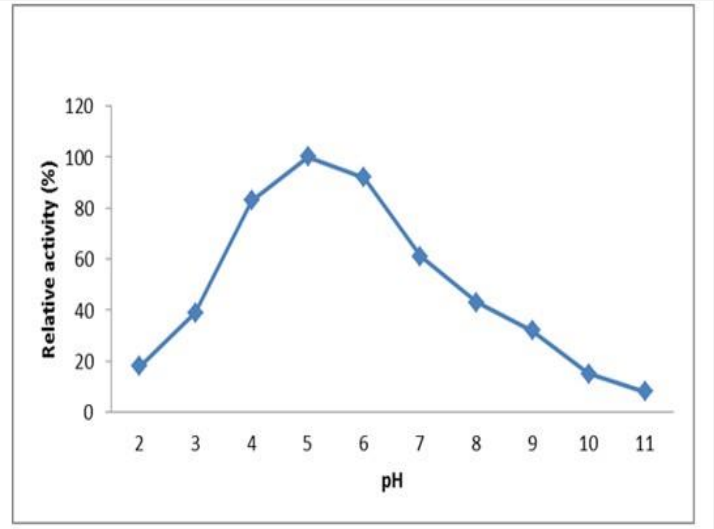


Figure 2: Effect of pH on the activity of

Effect of temperature on activity and stability of amylase:

To determine the effect of temperature on the activity of fungal amylase, the crude enzyme was mixed with starch soluble in 50 mM acetate buffer of pH 4.8 and incubated at temperatures of 20° C, 30° C, 40° C, 50° C, 60° C, 70° C, 80° C and 90° C for 10 min. Then the activity of amylase enzyme was determined by standard assay method Millern HP.

The result showed that as temperature increases amylase activity also increase at a certain temperature. The highest relative activity of amylase was observed in a range of 40-600C and reached its maximum activity at 500C. There was a decrease in enzyme activity when the incubating temperature was further increased beyond the maximum and the least activity also observed at 900C (Figure 4). Temperature above 50°C result in moisture loss of the substrate, affect metabolic activities, reduction in growth and enzyme production of microorganism.

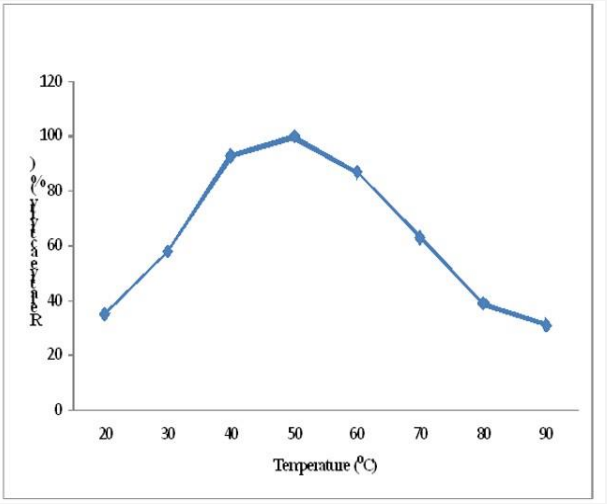


Figure 4: Effect of temperature on activity of

Effect of incubation period on amylase production:

Fungal amylase from selected isolate was produced by using solid-sate fermentation at different time intervals ranging from 24, 48, 72, 96, 120 and 144 hrs incubation. As the incubation time increase amylase activity also increase and maximum amylase production was obtained at 72 hours of incubation. Beyond this period relative activity of enzyme was decreased (Figure 7).

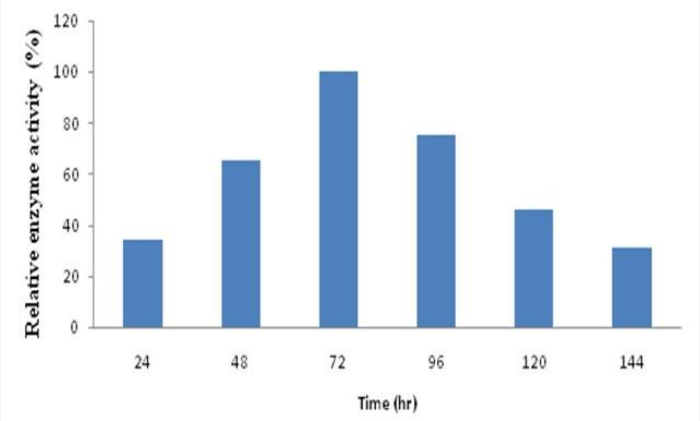


Figure 7: Effect of incubation period on Amylase

Effect of moisture level on production of amylase:

Enzyme production was affected by the moisture content of the substrate that was grown in SSF. To determine the maximum enzyme production the selected fungal isolate was grown on wheat bran containing different moisture contents. The result indicates that the least amount of amylase was produced in wheat bran substrate at moisture content of 45% and the maximum amylase yield was observed at 65% of moisture content. Increase in moisture content beyond 65% leads to reduction in product yield (Figure 8).

This is due to as moisture level increased anaerobic condition could be created, water molecules occupy the air space in the wheat bran, decreases porosity of solid state media, changes wheat bran particle structure, reduces gas volume, decreases diffusion and clumping of the solid particles resulted subsequent reduction in enzyme yield.

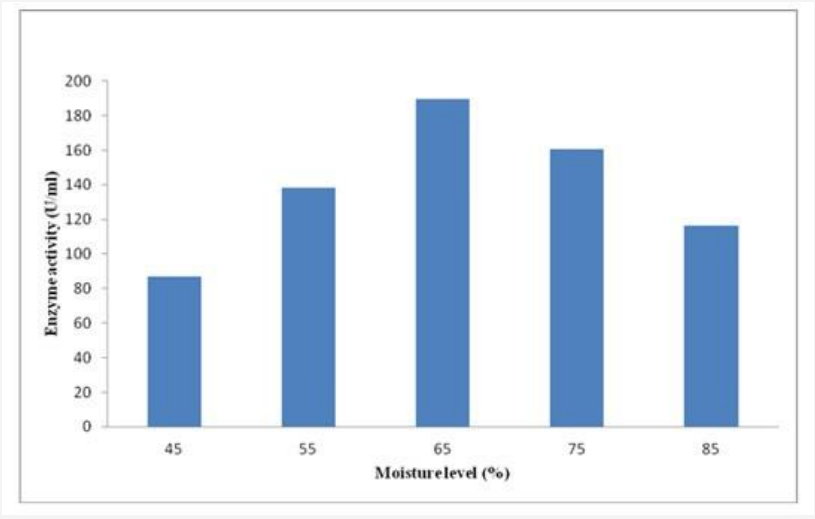


Figure 8: Effect of Moisture Level on Production of

Effect of carbon sources on amylase production:

The nature and amount of carbon source in culture media is important energetic source for the production of extracellular amylase from fungal isolate (Figure 9). Therefore, production of amylase was tested by growing in SSF containing different carbon source as a substrate. To determine amylase production supplementation of various carbon sources in growth media requires in the form of

monosaccharide's (Glucose and Fructose), disaccharides (Lactose and Sucrose) and polysaccharides (Starch and Wheat flour). flour was the best carbon source for amylase production whereas glucose results the least enzyme product and the other tested carbon sources gave comparatively less enzyme production, which is supplemented to the fermentation medium.

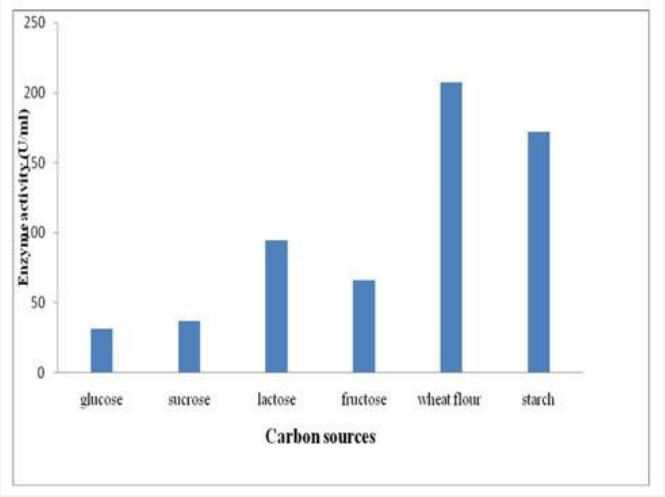


Figure 9: Effect of carbon sources on Amylase

Effect of nitrogen sources on amylase production:

Nitrogen is the secondary energy sources and plays an important role in the growth of the organism and enzyme production. The nature of the compound and the concentration that we used might stimulate or down reduce the production of enzymes. In this study the effect of supplementary organic nitrogen sources (Peptone, Tryptone, Urea) and inorganic nitrogen sources $(NH_4)_2SO_4$, NH_4NO_3, NH_4Cl) on the production of amylase by fungal strain using SSF was determined Among all the selected inorganic nitrogen sources maximum amylase production was obtained, when $(NH_4)2SO_4$ was added in the medium. In case of organic nitrogen sources peptone was found the highest for amylase production, probably due to its high content in minerals, vitamins, coenzymes and nitrogen components.

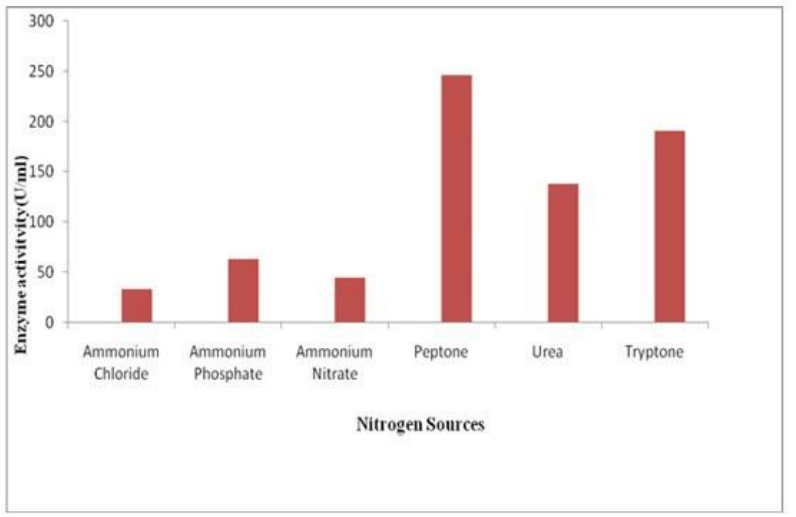
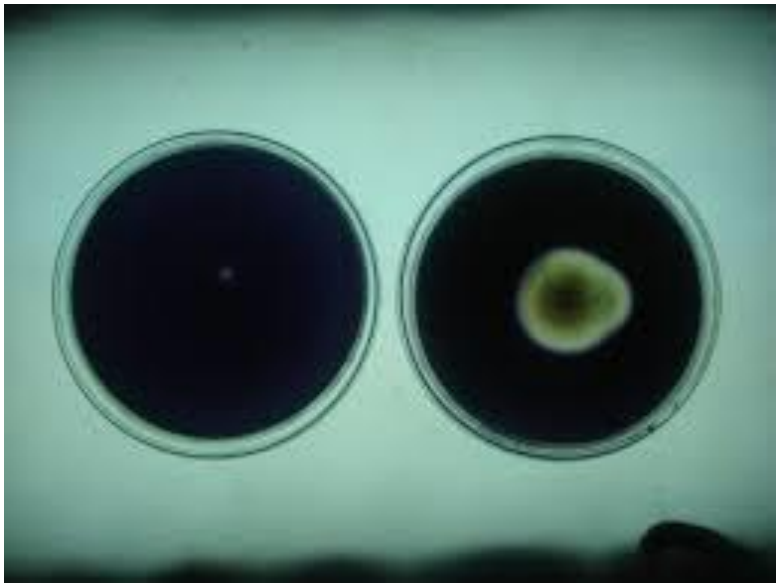


Figure 10: Effect of nitrogen sources on Amylase



Production of amylase by *A. niger*

Identification of industrial enzyme-producing microorganism

The identification of industrial enzyme-producing microorganisms, especially those which are non-toxic to human beings, are of high strategic interest. The industrial enzymes-producing bacteria are identified by morphological, cultural, microscopic, and biochemical characteristics as cleared in the Bergey's manual of determinative bacteriology.

The identity is further verified and confirmed by molecular techniques such as

16S rRNA or 16S rDNA sequence analysis. The industrial enzymes-producing fungi are traditionally identified on the basis of cultural, morphological and microscopic features. The identity is further confirmed by 18S rRNA sequence analysis in some cases (Table 1). Unlike for bacteria where the genomes of several bacteria have been sequenced, the fungal sequence analysis is at initial stages for fungi producing industrial enzymes. After rRNA / rDNA bacterial gene sequencing or 18S rRNA fungal molecular sequencing, the genomes have showed several new genes, and most of them were coding for industrial enzymes.

Industrial enzymes are generally produced under carefully controlled conditions by fermentation using microorganisms, especially bacteria or fungi. *Bacillus* and *Aspergillus* species were reported to be the main producers of industrial enzymes. Indeed, most of the species of these genera are safe and not producing any toxin, grow on inexpensive substrates and secrete extracellularly adequate amounts of enzymes in a reason. Recent developments in biotechnology are yielding efficient development of new enzymes particularly in the areas of protein engineering and directed evolution

production of Industrial enzymes

Markets for traditional industrial enzymes remain to grow while the continued prominence on biotechnological accomplishments has generated demand for an ever increasing number of additional biocatalysts. The emergences of genetic manipulations have now enabled the large-scale production of enzymes and other proteins which are produced naturally only in minute quantities. The level of downstream processing to which any enzyme is subjected is depending on its proposed application. Industrial enzymes produced in bulk usually need little downstream processing, and hence are relatively crude preparations .The production of commercial enzymes from fungi are 60% followed by 24% bacteria, 4% yeast, 2% Streptomyces, 6% higher animals, and 4% plants.

In earlier technologies animal and plant origin enzymes were largely used and till now for specific enzymes they are the main sources. The animal tissues and organs are very fine sources for enzymes such as proteases, lipases, and esterases for example, lysozyme is mostly get from hen eggs. Similarly, some enzymes are originated only from plants as brilliant sources such as papain (papaya) and bromelain (pineapple).

There are several down sides related with the enzymes making from plant and animal sources. Due to limited quantities there is a wide disparity in distribution. Apart from all the difficulties, the most important is the isolation, purification of the enzymes, and the cost factor as regard to industrial enzymes extracting from bovine source which contains heavy risk of contamination due to bovine spongiform encephalopathy (BSE is a prion disease occurred due to the ingestion of abnormal protein) therefore, microbial production of enzymes is used. There exists a likelihood of producing commercial enzymes straight by mammalian cell cultures. Other than the most important limit is the cost factor which is extremely high. Although some therapeutic enzymes are prepared through cell culture technique like tissue plasminogen, microorganisms are the most noteworthy and suitable source of commercial enzymes.

They can be made to prepare large amounts of enzymes under optimal growth conditions. Cultivation of microorganism by using low cost media as well as the growth of microorganism takes in short span of time. In addition, by using genetic engineering techniques on microorganism, desired product is produced. Isolation, purification, and recovery processes are easy with microbial enzymes as compared to plant and animal sources. In fact, most of the enzymes used in industrial processes are produced from microorganisms. Variety of fungi, bacteria, and yeast are produced for this purpose.

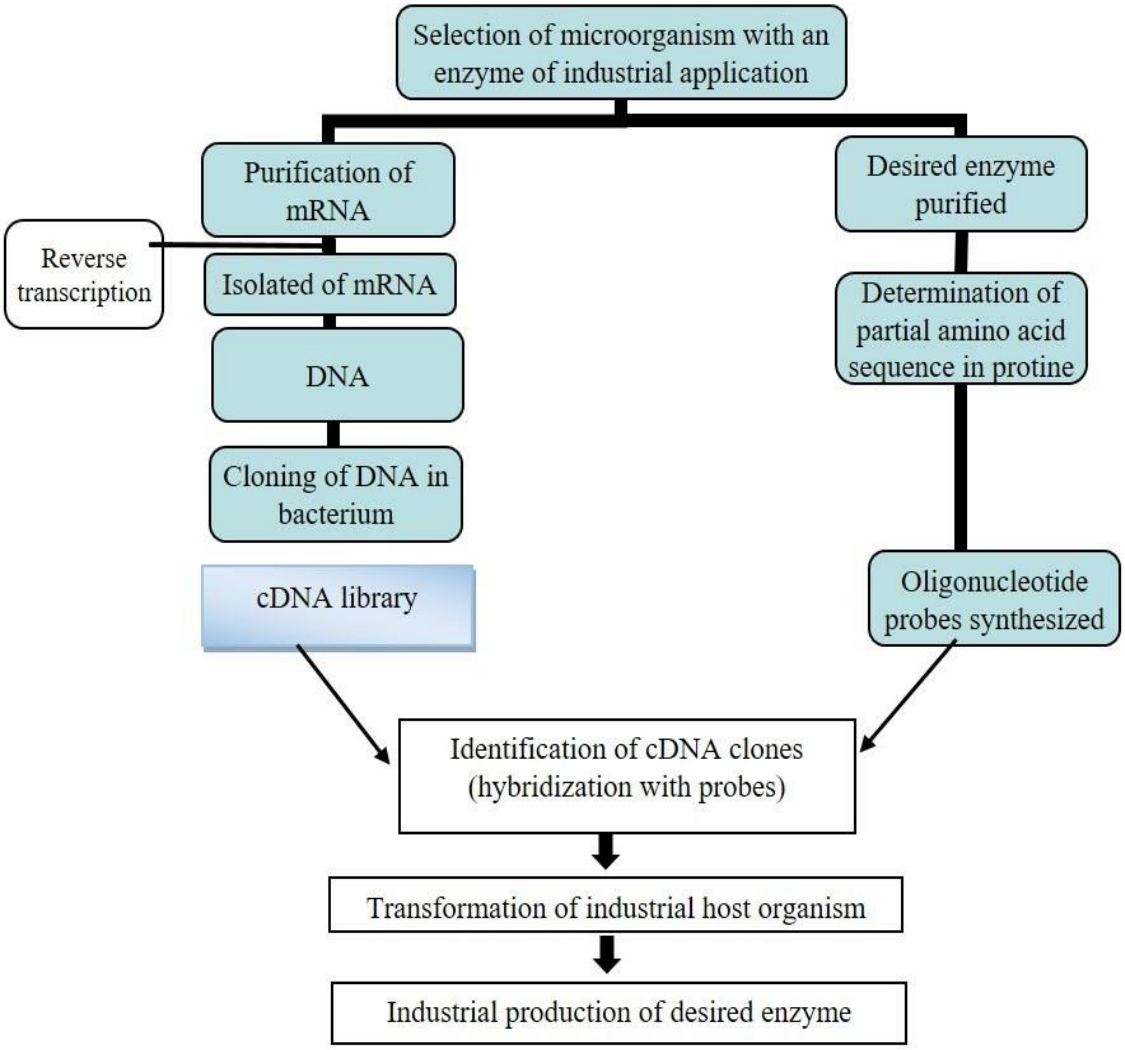


Fig. 2. Schematic representation of a cloning strategy for industrial production of enzymes.

Production of microbial industrial enzymes by microorganisms

The production of industrial enzymes from microorganisms involves various steps. These are isolation, screening and identification of enzymesproducing microorganisms, optimization of process parameters and fermentation for industrial enzyme production, purification and characterization of purified enzymes, industrial enzymes formulation for sale, customer liaison, and working with the regulatory authority bodies . Most of the bacteria and fungi used to produce industrial enzymes are genetically modified to overproduce them in significant amount.

Many enzymes are produced in industries but most predominant enzymes that are produced on large scale in industries include protease, alpha-amylase, glucose isomerase and glucamylase. Enzymes produced in industries with the help of microorganisms were found to exhibit good biological activity. Microbial source is preferred over plants and animals for production of enzymes mainly because of the following reasons

1) Enzymes can be produced on large scale and are economical.

2) The process of extraction and purification of enzymes from microbial sources is easier in comparison with plant

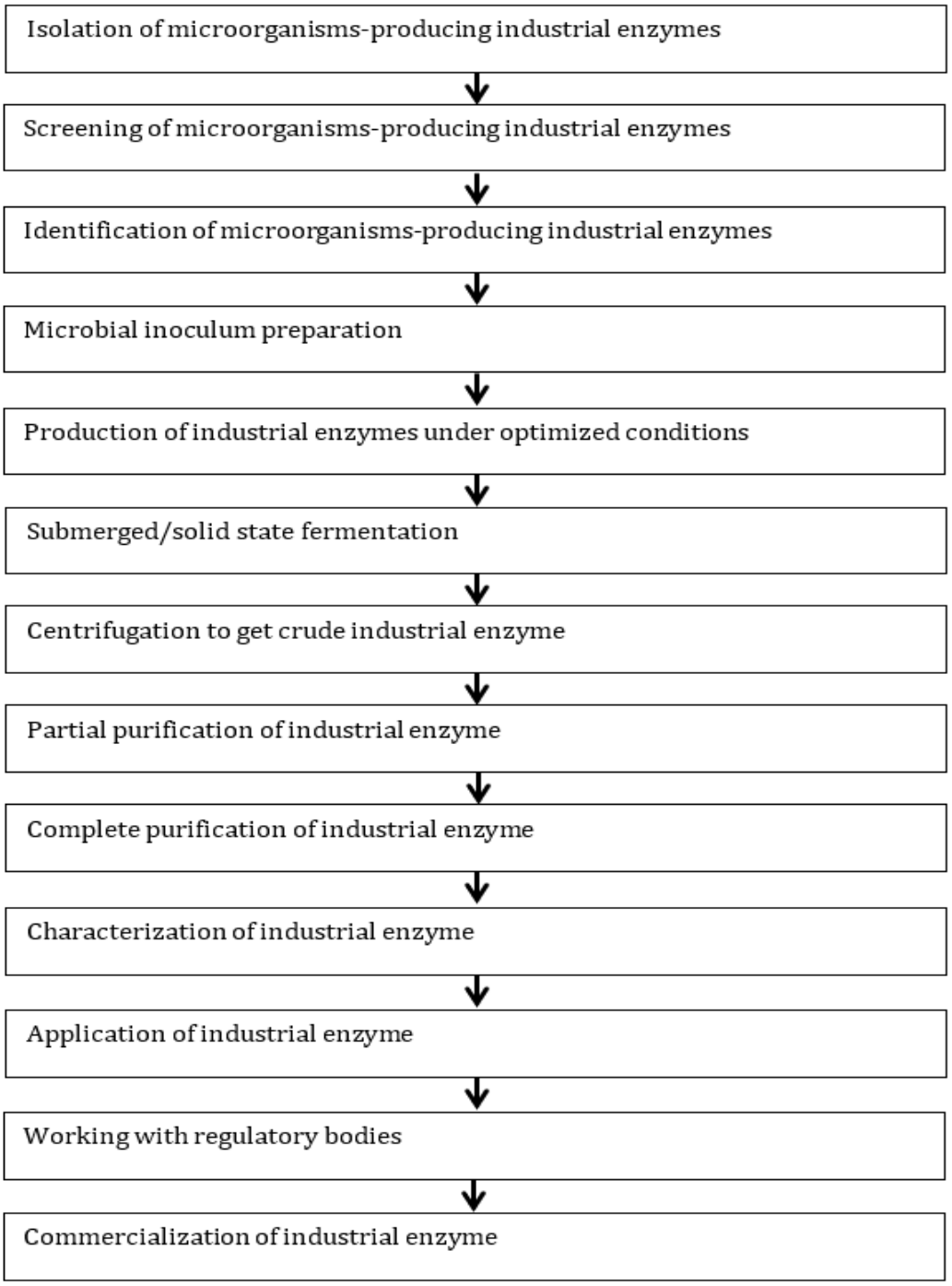
and animal sources.

3) Microbial sources are capable of producing variety of enzymes in different environmental conditions in limited

space and time period.

4) Genetic manipulation is carried out to yield higher quantity of enzymes produced from microbial sources

Solid state and submerged fermentation are often used to produce industrial enzymes. However, the submerged fermentation was repeatedly reported to be the method of choice for industrial enzymes secretion from microorganisms owing to the extracellular nature of the industrial enzyme that gets liberated in to the production media.



Fig(1): Production of industrial enzymes by microorganisms

Fermentation

Enzymes have been used for thousands of years to produce food and beverages, such as cheese, yoghurt, beer and wine. Yeast is a fungus whose enzymes aid the breakdown of glucose into ethanol and carbon dioxide anaerobically. The enzymes in yeast break down sugar (glucose) into alcohol (ethanol) and carbon dioxide gas:

Glucose $-$Yeast $- \rightarrow -$ Ethanol + Carbon dioxide

$C_6H_{12}O_6$ (aq) $-$Yeast $- \rightarrow -$ 2 C_2H_5OH (aq) + 2 CO_2 (g)

This reaction, which takes place in the absence of oxygen, is called fermentation.

Fermentation works best when the yeast and glucose solution is kept warm. Enzymes will also become ineffective if the temperature becomes too high. Fermentation is used in all production of alcoholic drinks. For stronger alcohol, such as whiskey and vodka, these need to be distilled after fermentation to increase the concentration of ethanol in the fermented mixture. This is due to the fact that ethanol poisons the yeast and stops it working when the concentration builds up about 18% by volume.

Fermentation is also used in the baking industry to make bread rise. After the dough has been prepared, it is left to rest in a warm place before going into the oven. This gives the enzymes in the yeast a chance to break down the sugar and make carbon dioxide.

Methods of fermentation

1- Submerged fermentation

Submerged fermentation is the cultivation of microorganisms in liquid nutrient broth. Industrial enzymes can be produced using this process. This involves growing carefully selected microorganisms (bacteria and fungi) in closed vessels containing a rich broth of nutrients (the fermentation medium) and a high concentration of oxygen. As the microorganisms break down the nutrients, they release the desired enzymes into solution.

Due to the development of large-scale fermentation technologies, the production of microbial enzymes accounts for a significant proportion of the biotechnology industries total output. Fermentation takes place in large vessels (fermenter) with volumes of up to 1,000 cubic metres.

The fermentation media sterilizes nutrients based on renewable raw materials like maize, sugars and soya. Most industrial enzymes are secreted by microorganisms into the fermentation medium in order to break down the carbon and nitrogen sources. Batch-fed and continuous fermentation processes are common. In the batch-fed process, sterilized nutrients are added to the fermenter during the growth of the biomass. In the continuous process, sterilized liquid nutrients are fed into the fermenter at the same flow rate as the fermentation broth leaving the system.

This will achieve a steady-state production. Parameters like temperature, pH, oxygen consumption and carbon dioxide formation are measured and controlled to optimise the fermentation process.

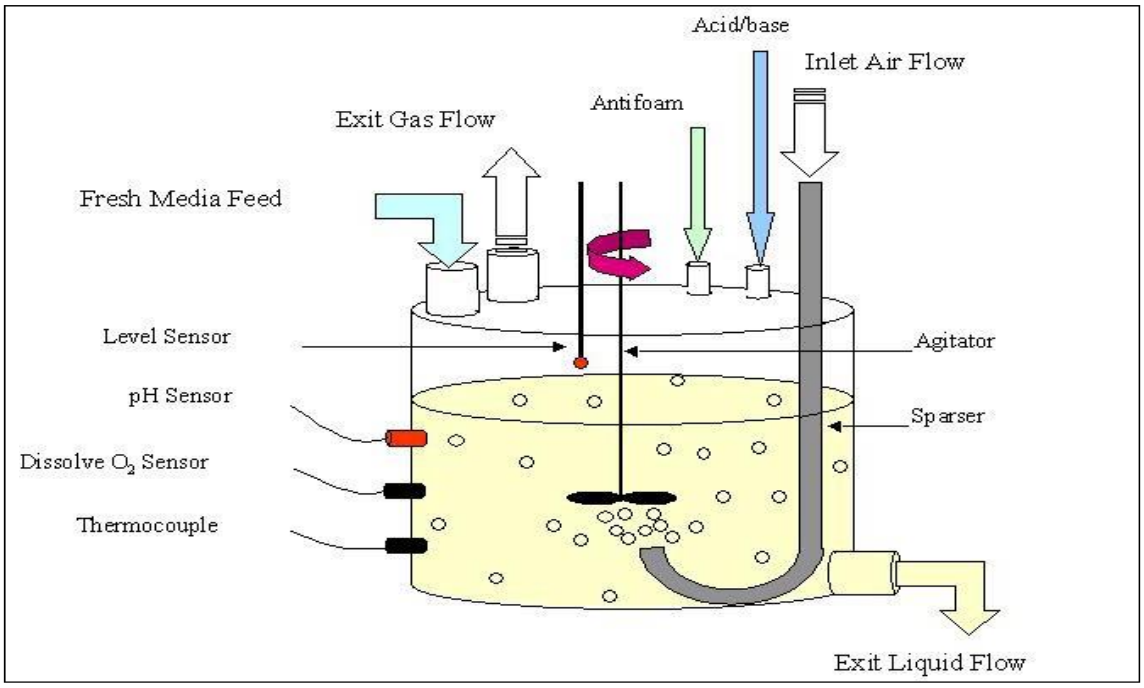


Fig. 1: Typical fermenter

Solid state fermentation

Solid-state fermentation (SSF) is another method used for the production of enzymes.

Solid-state fermentation involves the cultivation of microorganisms on a solid substrate, such as grains, rice and wheat bran. This method is an alternative to the production of enzymes in liquid by submerged fermentation. SSF has many advantages over submerged fermentation. These include, high volumetric productivity, relatively high concentration of product, less effluent generated and simple fermentation equipment.

There are many substrates that can be utilized for the production of enzymes by SSF. These include wheat bran, rice bran, sugar beet pulp and wheat and corn flour. The selection of substrate depends on many factors, which is mainly related to the cost and the availability of the substrate. Other factors include particle size and the

level of moisture. Smaller substrate particles have a larger surface area for the proliferation of the microorganisms, but if too small the efficiency of respiration will be impeded and poor growth and hence poor production of enzymes will result. Larger particles provide more efficient aeration and respiration, but there is a reduction in the surface area.

SSF requires moisture to be present on the substrate, for the microorganisms to produce enzymes. As a consequence the water content of the substrate must also be optimized, as a higher or lower presence of water may adversely affect the microbial activity. Water also has implications for the physicochemical properties of the solid substrate. Enzymes of industrial importance have been produced by SSF. Some examples are proteases, pectinases, glucoamylases and cellulases.

Types of fermentation process

Fermentation in liquid media is of two types depending upon the mode of operation:

A. Batch fermentation

B. Continuous fermentation

A. Batch fermentation

Batch reactors are simplest type of mode of reactor operation. In this mode, the reactor is filled with medium and the fermentation is allowed to proceed. When the fermentation has finished the contents are emptied for downstream processing. The reactor is then cleaned, re-filled, re-inoculated and the fermentation process starts again.

B. Continuous fermentation

Continuous reactors: Fresh media is continuously added and bioreactor fluid is continuously removed. As a result, cells continuously receive fresh medium and products and waste products and cells are continuously removed for processing. The reactor can thus be operated for long periods of time without having to be shut down. Continuous reactors can be many times more productive than batch reactors. This is partly due to the fact that the reactor does not have to be shut down as regularly and also due to the fact that the growth rate of the bacteria in the reactor can be more easily controlled and optimized.

Continuous reactors are as yet not widely used in industry but do find major application in wastewater treatment. Fed batch reactor is the most common type of reactor used in industry. In this reactor, fresh media is continuous or sometimes periodically added to the bioreactor but unlike a continuous reactor, there is no continuous removal. The fermenter is emptied or partially emptied when reactor is full or fermentation is finished. As with the continuous reactor, it is possible to achieve high productivities due to the fact that the growth rate of the cells can be optimized by controlling the flow rate of the feed entering the reactor.

Advantages and dis advantages of submerged fermentation (SMF) and/or solid state fermentation

For the industrial production of microbial enzymes, submerged fermentation (SMF) and/or solid state fermentation (SSF) are employed. Each fermentation has its advantges and disadvantages. The submerged liquid conditions are mostly preferred as compared to the solid-substrate fermentation because in the submerged culture methods the yields are more and contamination chances are less. It allows extracellular industrial enzyme secretion in important amounts in the production

medium and thus industrial enzyme recovery is high. In addition, the culture parameters are easily controlled. However, one of the disadvantages of SmF is that it uses expensive synthetic media.

However, historically solid-substrate fermentation is vital and immobile utilize for the fungal enzymes production for example, amylases, cellulases, proteases, and pectinases. . It uses inexpensive substances like agro industrial by products and downstream process is not expensive. The batch or continuous sterilization techniques are employed for medium sterilization. The growth conditions for fermentation viz., substrate, O2 supply, pH, and temperature are maintained at optimal levels after inoculating the medium with desire culture The froth formation can be controlled by adding antifoam agents and mostly the batch fermentation is used for production of enzymes, whereas continuous process used in lesser extent. Throughout the fermentation process the bioreactor system must be operated under sterile conditions..

Factors influencing fermentation

A fermentation is influenced by numerous factors, including temperature, pH, nature and composition of the medium, dissolved O2, dissolved CO2, operational system (e.g. batch, fed-batch, continuous), feeding with precursors, mixing (cycling through varying environments), and shear rates in the fermenter. Variation in these factors may affect: the rate of fermentation; the product spectrum and yield; the organoleptic properties of the product (appearances, taste, smell, texture), The generation of toxins; nutritional quality; and other physic-chemical properities.

1- Effect of time

The effect of incubation period on enzyme production was investigated by checking the enzyme activity on 4th, 5th, 6th, 7th and 8th days of incubation in the different substrates at pH 7 and at room temperature.

2- Effect of temperature

The effect of temperature on enzyme production was investigated by fermentation in different substrates and incubated at 30^{o} C, 37^{o} C, 40^{o} C, 45^{o} C and 50^{o} C at pH 7 for 6 days

3- Effect of pH

Fermentation investigated the effect of pH on enzyme production in different substrates by adjusting the pH of basal salt solutions to 4, 5.5, 6.5, 7, 7.5, 8 and 9. The substrates were then incubated for 6 days at room temperature.

4- Effect of carbon source

The effect of carbon sources on enzyme production was investigated by supplementing the basal salt solution, pH 7, with 2% of different carbon sources such as glucose, maltose, lactose, sucrose and starch. The substrates were then incubated for 6 days at room temperature.

5- Effect of nitrogen source

The effect of nitrogen source on enzyme production was studied by replacing the nitrogen source in basal salt solution, pH 7, with 2% of NaNO3, (NH4)2SO4, NH4Cl, NH4NO3 and KNO3, and incubated at room temperature for 6 days7 .

Optimization of process parameters for maximum industrial enzymes production by microorganisms

The production of some industrial enzymes is stimulated by various substances in the production medium. For instance, the production of the detergent lipase by Bacillus flexus XJU-1 was activated by Tween-80 and Triton X-100. Indeed, the present surfactants act by modifying plasma membrane thereby activating the medium compounds uptake, leading to the lipase release in a significant amount. The increase in extracellular cellulase secretion by *Penicillium* sp. When the surfactant Triton-X100 was supplemented to the fermentation medium. The production of laccase by a mushroom Stereum ostrea was stimulated by various inducers such as

aromatic or phenolic compounds, copper and surfactants. Indeed, the micronutrient copper activates laccase transcription and production, whereas surfactants favor enzyme production by stimulating spores growth and enhancing the availability of less soluble substrates for the microorganism

The industrial enzymes production by microorganisms is principally influenced by various factors such as incubation time, agitation/shaking, initial pH, inoculum concentration, incubation temperature, carbon source, metal ions, and nitrogen source. The optimization of these process factors has a significant role in enhancing the industrial enzyme yield. The optimization of media components, cultural parameters and fermentation conditions is therefore necessary to maximally produce the industrial enzymes in adequate amounts. The process factors are generally optimized one factor each time, holding all other factors unchanged and the optimized condition/factor is taken into account in the subsequent experiments in sequential order. The advantage of the optimization of various nutritional parameters, physico-chemical aspects, and fermentation factors is that it helps in designing a cost effective fermentation process.

Effect of incubation time on the industrial enzymes production by microorganisms

a- The incubation time of bacterial enzymes

The incubation time plays an important role in the production of industrial enzymes by bacteria and fungi. The optimal time recorded for industrial enzymes production mainly ranged from 24 to 48 h for bacterial species. The shorter incubation time reported for most of industrial enzymes production makes the fermentation process inexpensive. The incubation period of 60-96 h range was also reported for the production of industrial enzymes such as keratinase, protease, pullulanase, amylase and lipase by bacteria. A higher incubation period of 120 and 168 h were observed for cyclodextrin glycosyltransferase (or CGTase) production by

Bacillus flexus MSBC 2 and amylase secretion by *Streptomyces* strain A3, respectively. Therefore, the time period of industrial enzymes secretion by bacteria varies from one species

b- The incubation time of fungal enzymes

The fungal industrial enzymes are secreted at optimal level at 4 or 5th day .A low fermentation time of 2 and 3 days was observed for the production of the invertases from the *Saccharomyces cerevisiae* and L-asparaginase by *Trichoderma viride* Pers: SF Grey, respectively. The higher incubation time of 7 days was also noted for the production of pectinases by *Aspergillus niger*, cellulases by *Penicillium sp*. and *Aspergillus awamori* , tannase by *Mucor circinelloides* isolate and laccases by *Scytalidium lignicola*. Likewise, a higher incubation time of 12 days when laccase was secreted in an adequate amounts by *Stereum ostrea.*

In general, as the incubation time increases, the industrial enzymes secretion by microorganisms also increases. However, after optimum incubation period, a decline in industrial enzyme production is observed. This decrease in industrial enzymes production was attributed to the reduced availability of nutrients and the toxic metabolites secretion or decomposition of industrial enzyme by the protease For industrial lipase, the decrease was ascribed to the accumulation of fatty acids and glycerol resulted from lipolysis. For most industrial enzymes like amylases, the decrease in amylase production was due to the enzyme denaturation resulted from the enzyme produced and the medium components interaction.

2- Influence of initial pH of the medium on industrial enzymes production by microorganisms

The initial pH of the culture and fermentation medium is a major factor regulating industrial microbial enzymes secretion. It may influence the availability of nutrient substrates or the transport of various nutrient components across the bacterial or fungal membranes, which in turn stimulates the microbial growth and thus industrial enzymes production.

a- pH of bacterial enzymes

The optimum initial pH range recorded for most of the industrial bacterial enzymes is the 6 to 10 range. A low pH of 5.0 and 5.5 was seen for amylase production by *Chryseobacterium sp*. and phytase *by Bacillus lehensis* MLB2, respectively. 10.5 was higher pH recorded for industrial CG Tase production by *Bacillus halodurans.* The difference in genomes may also be the reason why the bacteria producing industrial enzymes have different initial pH requirements.

b- pH of fungal enzymes

The optimum pH observed for fungal industrial enzymes production ranges from acidic to basic pH range, viz. pH 5 to 9. Alkaline protease active at pH of 10.0 using *Aspergillus terreus* gr. Similary, lower pH values of 4 and 4.5 were observed as optimum initial pH in the production of tannase, phytase, and pectinases by *Aspergillus* species.

The variation in industrial enzyme yields at different initial pH requirement may be due to the bacterial or fungal strain specificity. Any deviation from optimum initial pH resulted in low industrial enzyme secretion. This was attributed to the disruption of transport mechanisms through the bacterial or fungal membrane that prevents the industrial enzyme release.

3-Effect of incubation temperature on the industrial enzymes production by microorganisms

The incubation temperature is a vital environmental parameter for industrial enzymes secretion by microorganisms. Like initial pH, it may influence the growth of bacteria and fungi, and thus industrial bacterial or fungal enzymes production.

a- Incubation temperature for bacterial enzymes

The optimum incubation temperature seen for bacterial industrial enzymes production is in the 30 to 50 °C range. 37 °C was noted as optimum production temperature in most cases. A low optimum incubation temperature of 20 and 28 °C were also observed for industrial amylase and lipase production, respectively by *Bacillus* species.

b- Incubation temperature for fungal enzymes

For fungal species, the optimum incubation temperature is ranged from 25 to 47 °C. However, 60 °C was optimum fermentation temperature for β-amaylase production by *Penicillium nigricans*. At elevated incubation temperature, the yield in industrial enzymes production is low due to the thermoliability of the industrial enzymes or the denaturing of industrial enzyme structure in the active site

4- Effect of inoculum level on the industrial enzymes production by microorganisms

The concentration of inoculum is one of the key culture parameters for microbial growth and thus industrial enzymes production. Various inoculum

concentrations ranging from 0.6 to 4% were optimum for bacterial industrial enzymes production by microorganisms. However, a detergent lipase production by *Staphylococcus arlettae* when a higher inoculum size of 10% was used.

Different inoculum levels were found to maximally produce the industrial enzymes by different fungi. For instance, the inoculum level of 2% was optimum for the tannase production by *Mucor circinelloides* isolate and protease production by *Aspergillus terreus* gr. 3% was inoculum size optimum for the secretion of a protease by *Scopulariopsis sp*. The effect of inoculum on industrial enzymes secretion may thus depend on the type of microorganism, inoculum load, and the bacterial or fungal size, and type.

Generally, an important increase in industrial enzymes production by microorganisms correlated with an enhancement in inoculum concentration till optimum inoculum size reached owing to rapid substrate degradation .Indeed, the industrial enzyme production by microorganisms is often high at lower inoculum levels; however, a low industrial enzyme yield is observed.

5- Effect of carbon source on industrial enzymes production by microorganisms

The carbon sources serve as a primary energy source for bacterial and fungal growth and therefore industrial enzymes production.

a- Effect of carbon source on bacterial enzymes

Glucose, soluble pullulan, malt extract, maltose, pullulan, sucrose and carboxymethylcellulose are used for the production of the bacterial industrial

enzymes. Soybean oil, olive oil and cotton seed oil were also used as carbon sources. In some cases, a mixture of carbon sources like corn flour and bean flour , glucose and skim milk and glucose and sesame oil are used in the secretion of bacterial enzymes.

b- EfFect of carbon source on fungal enzymes

For the production of industrial fungal enzymes, starch, glucose, maltose , sucrose, lactose, carboxymethylcellulose, pectin and tannate are employed as carbon sources. Various inexpensive substances such wheat bran, green tea leaves and orange peel moistened with molasses were preferred as carbon sources for industrial enzymes production by fungi.

The industrial enzymes are generally produced by microorganisms with low carbon source concentration. This make the production cost effective used. In some cases, a carbon source repression is observed when a carbon source is used in a significant amount. When an important amount of carbon source is utilized, industrial enzymes secretion decreased owing to limitation of oxygen transfer resulting in poor bacterial or fungal growth.

6- Effect of nitrogen sources on industrial enzymes production by microorganisms

he nitrogen sources served as a secondary energy source for the microbial growth and thus for industrial enzymes production. They play an important role in most of the microorganisms to synthetize the cell wall components, amino acids, peptides, proteins (including industrial enzymes), and nucleotides / nucleic acids.**s**

For the production of bacterial industrial enzymes, tryptone, peptone, yeast extract and beef extract are employed. Sometimes, the optimum industrial enzymes

production is observed when there is combinat of nitrogen sources, like peptone and yeast extract. The cheap organic nitrogen sources like corn flour and bean flour and soybean meal are also utilized to produce industrial enzymes by bacteria

Like for bacteria, organic nitrogen sources such as peptone, tryptone , urea yeast extract, beef extract and L-asparagine are used in industrial fungal enzymes production. In general, the industrial enzymes are generally produced at their optimum levels when organic nitrogen sources are incorporated in the production medium. The preference of organic nitrogen sources by industrial enzymes producing microorganisms can be ascribed to the presence of some micro-and micronutrients, vitamins, amino acids and/or peptides, and growth factors present in them.

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Although the inorganic nitrogen sources are not generally often found to increase the production of the industrial enzymes, an important industrial enzymes production was observed in some cases with them. For instance, a significant industrial enzyme production was seen when ammonium acetate, potassium nitrate and ammonium sulfate were used from industrial production by bacteria. Similarly, ammonium nitrate, potassium nitrate, sodium nitrate and sodium nitrite were inorganic nitrogen sources used to produce industrial enzymes by fungi.

7- Effect of agitation on the industrial enzymes production

The industrial enzymes-producing microorganisms are usually grown under shaking conditions. The production of bacterial industrial enzymes is often done with shaking in the range of 100 to 200 rev/min. For instance, 100 rpm was the optimum for the production of industrial enzymes by *Bacillus* species, while 200 rpm was also best for some *bacterial* species. Similarly, the production of industrial enzymes by fungi was agitated in the 120 to 200 rev/min range. For examples, 120 and 200 rpm were found as optimal shaking conditions for fungal species. The agitation of the culture flasks at a moderate rate allows a good availability of the nutrients to the microorganisms and a proper aeration, favoring thus the production of industrial enzymes in important amounts.

Methods of strain improvement

Microorganisms are used as source for production of enzymes, biomolecules and proteins in industries. Few examples of source of microorganism include *Saccharomyces cerevisiae* and *Aspergillus niger* are widely used in industries for production of enzymes and alcohol. A wild type strain is isolated for process of strain improvement and to increase productivity. To achieve growth rate faster, desirable downstream processing and behavior of fermentor is enhanced by altering cellular genetics and also it is important to understand the fundamentals of physiology and structure of organism.

The strategies differ from each source of microorganism for example in case of fungal source the emphasis is more on porosity of cell wall, differentiation, secretion and branching.

Whereas in case of yeast fermentation process involves gene regulation and ploidy through which carbon sources will play apredominant role in production of proteins associated with heterologous gene expression. Wild types of strains which are used for producing metabolic concentrations are not economical. Improvement of strains is considered as cost effective process and it is necessary to produce secondary metabolites

Desirable strain isolation depends on system and they exhibit following features like. Rapid growth, Genetic stability, Non toxic to humans, Large sized cells, Fermentation process time is less and Exhibit tolerance to carbon or nitrogen sources present in higher concentrations. Few methods that are associated with strain improvement process are Recombinant DNA technology, Recombination Protoplast fusion and Mutations-Site-directed mutagenesis.

The successful application of these methods is enhanced by increasing a dose of gene concentration will increase the product activity which includes one or more number of genes, for example enzymes. Recent developments in biotechnology are yielding efficient development of new enzymes particularly in the areas of protein engineering and directed evolution

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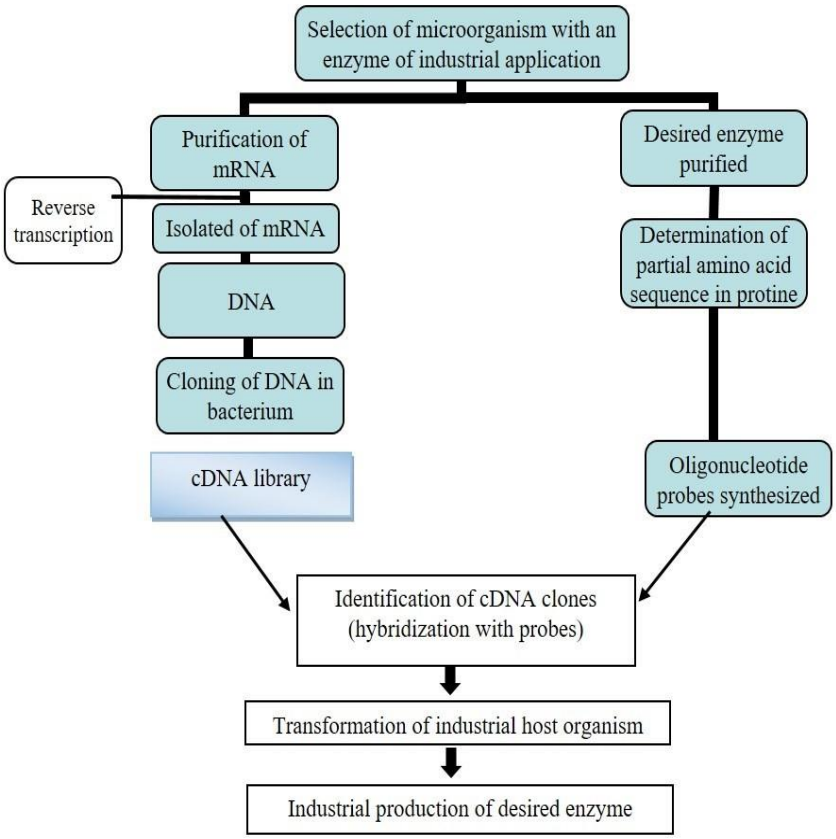

Fig. 2. Schematic representation of a cloning strategy for industrial production of enzymes.

1. Improvement of enzyme recovery and enzyme activity

The membranes are now considered as critical part of enzyme recovery process and it is replacing conventional downstream processing approaches to purify and recovery of enzymes in industries. Membrane filtration technology is considered as most efficient and economical in comparison with traditional approaches. Chemical treatment is replaced by membranes to minimize the costs of analytical, chemical and labor. Membranes are used to achieve optimum yield of enzymes which is very difficult to obtain by application of fermentation derived chemicals such as vitamins, polymers, enzymes and amino acids.

2-Membrane filtration technology

Membrane filtration technique augmented downstream processing is employed to remove the impurities to recovery of enzymes. The use of microfiltration, nanoflitration and ultra filtration membranes facilitated in complete removal of impurities and increased the amount of enzyme recovery. The application of membrane filtration technology to purify enzymes is currently used in industries for large scale production of desired enzymes and also to increase the percentage of enzyme recovery and activity .

Microfiltration is employed for purification of macromolecules from other molecules like cell debris or proteins. Ultrafiltration and nanofiltration were used during fermentation method involving downstream processing to retain the substrate and enzyme in reactors and remove the reaction inhibiting components. The advantages of using membrane filtration technology over traditional methods include

1) Membrane filtration technique can be operated at high Temperatures.

2) Greater efficiency in terms of energy and less time taking Process.

3) Technique is designed according to the specific product

requirement

4) Continuous fermentation is preferred over batch fermentation.

5) Cost reliable technique.

Enzymes purification

Enzyme purification is vital for the characterization of the function, structure and interactions of the protein of interest. The purification process may separate the enzyme and non-enzyme parts of the mixture, and finally separate the desired enzyme from all other enzymes. Separation of one enzyme from all others is typically the most laborious aspect of enzyme purification. Separation steps usually exploit differences in protein size, physico-chemical properties, binding affinity and biological activity. The pure result may be termed **enzyme isolate**.

Objectives of enzyme purification

- maximum possible yield + maximum catalytic activity + maximum possible purity „ Assay procedure „
- History „
- Crystallization „
- Homogenization + large scale separation „
- Attach the affinity tag to enzyme using DNA recombinant technology (ex. (His)6-tag).

Microbial Enzymes Extraction

If the protein of interest is not secreted by the organism into the surrounding solution, the first step of each purification process is the disruption of the cells containing the protein. Depending on how fragile the protein is and how stable the cells are, one could, for instance, use one of the following methods: i) repeated freezing and thawing, ii) sonication, iii) homogenization by high pressure (French press), iv) homogenization by grinding (bead mill), and v) permeabilization by detergents (e.g. Triton X-100) and/or enzymes (e.g. lysozyme). Finally, the cell debris can be removed by centrifugation so that the proteins and other soluble compounds remain in the supernatant.

Also proteases are released during cell lysis, which will start digesting the proteins in the solution. If the protein of interest is sensitive to proteolysis, it is recommended to proceed quickly, and to keep the extract cooled, to slow down the digestion. Alternatively, one or more protease inhibitors can be added to the lysis buffer immediately before cell disruption. Sometimes it is also necessary to add DNAse in order to reduce the viscosity of the cell lysate caused by a high DNA content.

Purification strategies

1.Precipitation and differential solubilization:

Ammonium sulfate precipitation

In bulk protein purification, a common first step to isolate proteins is precipitation with ammonium sulfate $(NH_4)_2SO_4$. This is performed by adding increasing amounts of ammonium sulfate and collecting the different fractions of precipitate protein. Ammonium sulfate can be removed by dialysis. The hydrophobic groups on the proteins get exposed to the atmosphere, attract other protein hydrophobic groups and get aggregated. Protein precipitated will be large enough to be visible. One advantage of this method is that it can be performed inexpensively with very large volumes.

The first proteins to be purified are water-soluble proteins. Purification of integral membrane proteins requires disruption of the cell membrane in order to isolate any one particular protein from others that are in the same membrane compartment. Sometimes a particular membrane fraction can be isolated first, such as isolating mitochondria from cells before purifying a protein located in a mitochondrial membrane.

2. Sucrose gradient centrifugation

A linear concentration gradient of sugar (typically sucrose, glycerol, or a silica based density gradient media, like Percoll) is generated in a tube such that the highest concentration is on the bottom and lowest on top. Percoll is a trademark owned by GE Healthcare companies. A protein sample is then layered on top of the gradient and spun at high speeds in an ultracentrifuge. This causes heavy macromolecules to migrate towards the bottom of the tube faster than lighter material. During centrifugation in the absence of sucrose, as particles move farther and farther from the center of rotation, they experience more and more centrifugal force (the further they move, the faster they move)

3. chromatographic methods

Many different chromatographic methods exist :

a. Size exclusion chromatography :

Also known as molecular sieve chromatography, is a chromatographic method in which molecules in solution are separated by their size, and in some cases molecular weight. It is usually applied to large molecules or macromolecular complexes such as proteins and industrial polymers. Typically, when an aqueous solution is used to transport the sample through the column, the technique is known as gel-filtration chromatography

b. Ion exchange chromatography :

Is separates compounds according to the nature and degree of their ionic charge. The column to be used is selected according to its type and strength of

charge. Anion exchange resins have a positive charge and are used to retain and separate negatively charged compounds (anions), while cation exchange resins have a negative charge and are used to separate positively charged molecules (cations).

c. Affinity chromatography :

Is a separation technique based upon molecular conformation, which frequently utilizes application specific resins. These resins have ligands attached to their surfaces which are specific for the compounds to be separated. Most frequently, these ligands function in a fashion similar to that of antibody-antigen interactions. This "lock and key" fit between the ligand and its target compound makes it highly specific, frequently generating a single peak, while all else in the sample is unretained.

d. High performance liquid chromatography

High performance liquid chromatography or high pressure liquid chromatography is a form of chromatography applying high pressure to drive the solutes through the column faster. This means that the diffusion is limited and the resolution is improved. The most common form is "reversed phase" HPLC, where the column material is hydrophobic. The proteins are eluted by a gradient of increasing amounts of an organic solvent, such as acetonitrile.

The enzymes produced by fermentation

The enzymes produced by fermented by nearly all living cells for catalysis of their own specific biochemical reactions in the metabolic process. Enzymes are playing an important role in food processing techniques for improving nutritive value and flavor of processed food. The food processing industry the making of cheese, leavened bread, wine and beer, yogurt, and syrup is successfully using enzymes at the commercial level.

1**. α-Amylase**

Amylase enzymes hydrolyze complex starch molecules into simple monomer units of glucose. Sources of αamylase are plants, animals and microorganisms, but commercially viable amylases are produced from microorganisms, especially bacterial and fungal species. Thermostable α amylase is produced by some potential bacterial species like *Bacillus licheniformis* and *Bacillus stearothermophillus*, Pseudomonas, and the Clostridium family.

Starch-converting properties of α-amylases are playing an important role in the food, beverage, and sugar industries. α-Amylase is improving the quality of breads that have reduced size and poor crust color, and compensates for the nutritional deficiencies of the grain. α-Amylase also degrades the starch in wheat flour into small dextrins, thus allowing yeast to work continuously during dough fermentation, proofing, and the early stages of the baking process. α-Amylases are also employed in many other aspects of the food industry like clarification of beer, fruit juices, and pretreatment of animal feed to improve the digestibility of fiber. maltose (saccharifying amylase) Dextrins dextrinase maltose Starch or dextrins amyloglucosidase glucose.

Starch —(a-amylase / liquefying amylase)→ dextrins + maltose

Starch —(B-amylase / liquefying amylase)→ maltose

Dextrin —(dextrinase)→ maltose

amyloglucosidase

Starch or dextrine  glucose

Bacterial Amylases

Amylase can be obtained from different species of microorganisms, but for commercial use, *α*-amylase derived from *Bacillus licheniformis*, *Bacillus stearothermophilu*s, and *Bacillus amyloliquefaciens* has number of application in different industries such as in food, fermentation, textiles and paper industries. *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* are found to be good producers of thermostable *α*- amylase and are widely in use for commercial production of the enzyme for numerous applications. Today, thermostable amylases of *Bacillus stearothermophilus* or *Bacillus licheniformis* are used in starch processing industries

Fungal Amylase.

Most of the mesophilic fungi are reported to produce *α*-amylase, and many researches have been done for specific cultural conditions and to choose the best strains to produce commercially. Fungal enzymes are limited to terrestrial isolates, mostly to *Aspergillus* and *Penicillium*. The *Aspergillus* species usually produces a variety of extracellular enzymes, and amylases are the ones with the most significant industrial value. Filamentous fungi, such as *Aspergillus oryzae* and *Aspergillus niger*, produce large quantities of enzymes that can be used extensively in the industry.

Application of Amylase.

Amylases have a wide range of application in various industries such as in the food, bread making, paper industries, textiles, sweeteners, glucose and fructose syrups, fruit juices, detergents, fuel ethanol from starches, alcoholic beverages, digestive aid, and spot remover in dry cleaning. Bacterial *α*-amylases are also being used in clinical, medicinal, and analytical chemistry.The widely used thermostable

enzymes in the starch industry are the amylase.

Use in Starch Industry

The starch industry has the most widespread applications of amylases, which are used during starch hydrolysis in the starch liquefaction process that converts starch into fructose and glucose syrups. The enzymatic conversion of all starch includes gelatinization, which involves the dissolution of starch granules, thereby forming a viscous suspension; liquefaction, which involves partial hydrolysis and loss in viscosity; and saccharification, involving the production of glucose and maltose via further hydrolysis.

Use in Detergent Industry.

Both in terms of volume and value detergent industry are the primary consumers of enzymes. The application of enzymes in detergents making enhances the detergents ability to remove tough stains and also makes detergent ecofriendly. Amylases are the second type of enzymes used in the detergent formulation, and 90% of all liquid detergents contain these enzymes .These enzymes are used for laundry and automatic dishwashing to clean up residues of starchy foods such as custard, gravies, potato, and chocolate. and other smaller oligosaccharides.

Use in Food Industry.

There is an extensive use of amylase in processed food industry such as baking, brewing, production of cakes, preparation of digestive aids, fruit juices, and starch syrups. The α-amylases have been used in the baking industry widely These enzymes are generally added to the dough of bread in order to degrade the starch into smaller dextrins, which are further fermented by the yeast. The α-amylase enhances the fermentation rate and the reduction of the viscosity of dough, which results in improvements in the volume and texture of the product.

Use in Textile Industry

Amylases are utilized for desizing process in textile industry. Sizing agents like starch are added to yarn before fabric production for fast and secure weaving process. Starch is a very attractive size, because it is cheap, easily available all over, and it can be easily removed. Desizing is the process where removal of starch from the fabric takes place and acts as the strengthening agent to prevent breaking of the warp thread during the weaving process. The α-amylases selectively remove, the size and do not affect the fibres. For a long time amylase from Bacillus strain was employed in textile industry.

Use in Paper Industry.

The main use of α-amylases in the pulp and paper industry is the modification of starch of coated paper, that is, for the production of low-viscosity, high-molecular weight starch The coating treatment makes the surface of paper smooth and strong to improve the writing quality of the paper. For paper sizing the viscosity of natural enzyme is too high, and this can be changed by partially degrading the polymer with α-amylases in a batch or continuous processes. Starch is considered to be the good sizing agent for the finishing of paper, improving the quality and reusability, besides being a good coating for the paper.

Use in Medicine

A higher than normal concentration of amylases may predict one of several medical conditions, including acute inflammation of the pancreas, perforated peptic ulcer, strangulation ileus, torsion of an ovarian cyst, macroamylasemia, and mumps. In other body fluids also amylase can be measured, including urine and peritoneal fluid. In various human body fluids the level α- amylase activity is of clinical importance, for example, in diabetes, pancreatitis, and cancer research.

\Lipase.

It is an enzyme that catalyzes the breakdown or hydrolysis of fats. Lipases are a subclass of the esterases. Lipases catalyze the hydrolysis of ester bonds in lipid substrates and play a vital role in digestion and the transport and processing of dietary lipids substrate. Lipases catalyze the biochemical reaction like esterification, interesterification, and transesterification in nonaqueous media which frequently hydrolyze triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol. Lipases perform essential roles in the digestion, transport, and processing of dietary lipids (e.g., triglycerides, fats, and oils) in most, if not all, living organisms. Lipases are considered as major group of biotechnologically valuable enzymes, mainly due to the versatility of their applied properties and easy mass production.

Bacterial Lipase. Some of the lipase-producing bacterial genera include Bacillus, Pseudomonas, and Burkholderia. The commercially important bacterial lipases are usually extracellular, and also their bulk production is much easier. There are a number of lipase-producing bacteria, but only a few are commercially exploited as wild or recombinant strains.

Fungal Lipase. Fungi capable of synthesizing lipases are found in several habitats, including soils contaminated with wastes of vegetable oils, dairy byproduct, seeds, and deteriorated food .Candida rugosa lipases have been known for their diverse biotechnological potential.Other major lipaseproducing fungi are *Mucor*, *Candida*, *Penicillium*, *Rhizopus*, *Geotrichum*, *Rhizomucor*, *Aspergillus*, *Humicola*, and *Rhizopus*.

Use in Textile Industry.

In the textile industry lipases are used for the removal of size lubricants, which increases fabrics absorbance ability for improved levelness in dyeing. In the denim abrasion systems, it is used to lessen the frequency of cracks and streaks. Commercial preparations used for the desizing of denim and other cotton fabrics

contain both alpha.

Use in Detergent Industry.

The hydrolytic lipases are commercially very important, and their addition to detergents is mainly used in laundries and household dishwashers. Enzymes reduce the environmental load of detergent products, as they save energy by enabling a lower wash temperature to be used, and use of chemicals in detergents is reduced, mostly biodegradable, leaving no harmful residues has no negative impact on sewage treatment processes; and does not possess any kind of risk to aquatic life.

Use in Food Industry.

To modify the food flavour by synthesis of esters of short-chain fatty acids and alcohols (flavour and fragrance) lipases have been frequently used. Lipases play a major role in the fermentative steps during manufacturing of sausage and also to measure changes in long-chain fatty acid liberated during ripening. Previously, lipases of different microbial sources were used for refining rice flavour, modifying soybean milk, and for enhancing the aroma and speed up the fermentation of apple wine. By adding lipases the fat is removed while processing meat and fish, and this process is called biolipolysis.

Use in Diagnosis.

Lipases are considered as important drug targets or marker enzymes in the medical field. The presence or high levels of lipases can indicate certain infection or disease and can be used as diagnostic tool. They are used in the determination of serum triglycerides to liberate glycerol which is determined by enzyme-linked colorimetric reactions. Acute pancreatitis and pancreatic injury can be determined by the level of lipases in blood. Lipases can be used in the treatment of malignant tumors as they are the activators of tumor necrosis factor.

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Use in Medical Applications

Lipases isolated from Galleria mellonella (wax moth) were found to have a bactericidal action on Mycobacterium tuberculosis . This preliminary research may be considered as part of global unselected screening of biological and other samples for detecting new promising sources of drugs.

Use in Cosmetics.

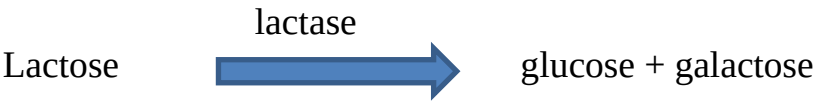
Retinoids (vitamin A and derivatives) are commercially very important in cosmetics and pharmaceuticals such as skin care products. Immobilized lipases are used for the preparation of water-soluble retinol derivatives. Lipases are used in hair waving preparation and have also been used as an ingredients of topical antiobese creams or as oral administration.

2. Lactase

Lactase enzymes catalyze the breakdown of the milk sugar lactose into simple sugar monomer units like glucose and galactose. Lactases are obtained from plants, animal, bacteria, fungus, yeasts and molds. Commercial production of lactase enzymes is developed from *Aspergillus niger*, *A. oryzae*, and *Kluyveromyces lactis* Fungal origin lactases have optimum activity at acidic pH ranges, and yeast and bacterialoriginated lactases have optimum pH ranges near to neutral. The lactase enzyme is predominantly rich in infancy and is called a brush border enzyme. Some people do not produce enough of the lactase enzyme so they do not properly digest milk.

This is called lactose intolerant, and people who are lactose intolerant need to supplement the lactase enzyme to aid in the digestion of milk sugar. Another useful application of the lactase enzyme is it increases the sweetness of lactase-treated

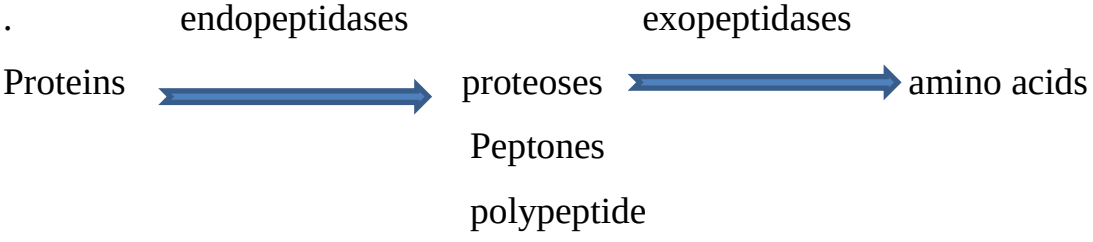
milk, and assists in the manufacturing of ice cream and yogurt preparation



Protease

Proteolytic enzymes are also termed as peptidases, proteases, and proteinases, which are able to hydrolyze peptide bonds in protein molecules. Proteases are generally classified as endopeptidases and exopeptidases. Exopeptidases cut the peptide bond proximal to the amino or carboxy termini of the protein substrate, and endopeptidases cut peptide bonds distant from the termini of the protein substrate. Proteases are obtained from diverse groups of organisms such as plants, animals, and microorganisms, but commercially viable proteases are obtained from microorganisms, especially bacterial and fungal species.

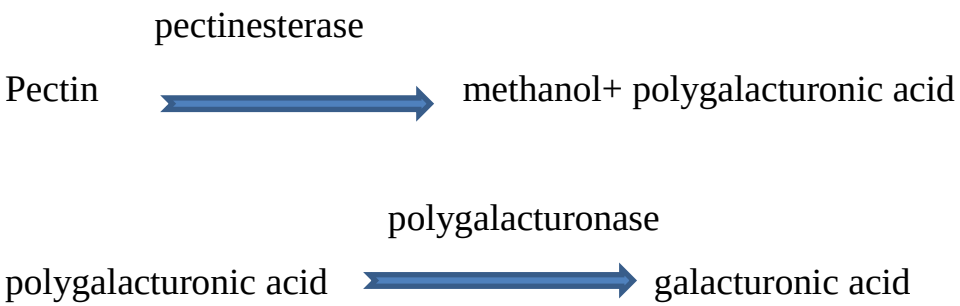
Microorganisms secrete the extracellular and intracellular proteases in both the submerged and solid-state fermentation process. Bacillus species of bacteria, like *Bacillus licheniformis*, *Bacillus subtilis*, and *Aspergillus* species of fungus like *Aspergillus niger*, *A. flavus*, A. fumigatus, A. oryzae, are the best sources of protease enzyme. Broad working range of temperature (10–80°C) and pH (4–12) of protease enzymes increases their application in the foodprocessing industry, the major role in cheese and dairy product manufacturing. Aminopeptidases are significantly improving the flavor in fermented milk products. Other basic applications of proteases in the foodprocessing industry are to increase the nutritive value of bread, baked goods, and crackers.



Pectinase

Pectinase breaks down pectin components, which are found in the middle lamella of plant cell walls. Pectin is made up of complex colloidal acid polysaccharides with a back bone of galacturonic acid residue with a α-1-4 linkage. Pectinase therefore helps to break down plant cell walls to extract cell sap. Potential microbial strains like *Moniliella* SB9, *Penicillium* spp. and *Aspergillus* spp. are good sources of commercial pectinase. Where, Dupaign, (1974) mention that the Pectinases are now an essential part of the fruit juice industry, as well as having various biotechnological applications in the fermentation of coffee and tea, the oil extraction processes, and the treatment of pectic waste water from the fruit juice industry.

Pectinase is lowering down the viscosity of fruit juice during the clarification process through the degradation of pectin substance in fruit juice and getting better pressing ability of pulp, simultaneously jelly structure are breaking down and increases the yields of fruit juice. Another significant application of pectinase enzymes in industrial processes is the refinement of vegetable fibers during the starch manufacturing process, such as the curing of coffee, cocoa and tobacco, canning of orange segments, and extracting sugar from date fruits.



Laccase

Laccase enzymes were first obtained from the cell sap of the Japanese lacquer tree. Laccase enzymes are isolated from plants, bacteria, fungi, and insects. Laccase

is responsible for discoloration, haze, wine stabilization, baking, and flavoring in food processing. Laccase improves the baking process through an oxidizing effect, and provides an additional development in the strength of dough and baked products, including enhancing crumb structure and increasing softness and volume. Another diverse application of laccase is in environmental sectors, which degrade various ranges of xenobiotic compounds

Xylose (Glucose) Isomerase

Xylose isomerase (d-xylose ketol-isomerase) catalyzes the isomerisation reaction of D-xylose into xylulose. This is initial step of xylose metabolism in microbial cell physiologies. Xylose isomerases are also referred to as glucose isomerases because of their capability to exchange d-glucose into d-fructose. Microorganisms are most suitable sources of xylose isomerase; some potential microbial species are *Streptomyces olivochromogenes*, *Bacillus stearothermophilus*, *Actinoplanes missouriensis*, known xylose isomerase procurers. Xylose isomerase loses its catalytic activities up to 50% under acidic conditions. The greatest application for glucose isomerase is in the foodprocessing industry; it mainly catalyzes two significant reactions such as reversible isomerization of d-glucose to d-fructose, and d-xylose to d-xylulose.

Catalase

Catalase enzymes break down hydrogen peroxide (H_2O_2) to water and oxygen molecules, which protects cells from oxidative damage by reactive oxygen species. Commercial catalases are produced from *Aspergillus niger* through a solid-state fermentation process. The major applications of catalase in the food-processing industry include working with other enzymes like glucose oxidase, which is useful in food preservation and egg processing, and sulphydryl oxidase, which under aseptic conditions, can eliminate the effect of volatile sulphydryl groups, that is, they generate from thermal induction and are responsible for the cooked/off-flavor in

ultra-pasteurized milk. Glucose Oxidase The glucose oxidase enzyme is commercially produced from *Aspergillus niger* and *Penicillium glaucum* through a solid-state fermentation method. Muller was the first one who reported that the catalyzation of glucose oxidase and the breakdown of glucose into gluconic acid in the presence of dissolved oxygen. Fungal strains *Aspergillus niger* are able to produce notable amounts of glucose oxidase. Glucose oxidase enzymes are used to remove small amounts of oxygen from food products or glucose from diabetic drinks. Glucose oxidase is playing an important role in color development, flavor, texture and increasing the shelf life of food products.

$$2\ H_2O_2 \xrightarrow{\text{Catalase}} 2H_20 + 0_2$$

$$2C_6H_{12}0_6 + 0_2 + H_20 \xrightarrow{\text{Glucose oxidase}} C_6H_{12}0_7 + H_20_2 \text{ (gluconic acid)}$$

Transglutaminase

Transglutaminase enzymes catalyze reactions to alter proteins by merging amine, crosslinking, and deamination. Transglutaminase is responsible for acyl transfer, deamidation, and the inter- and intra-molecular crosslink between amino acid residues of glutamine and lysine. The commercial application of transglutaminase enzymes in the food-processing industry is improving the protein-emulsifying capacity, gelatation, viscosity, and production of various types of protein ingredients to enhance the quality of food products. Transglutaminase is enhancing the water-holding capacity, softness, foam formation, and stability of food products. Extracellular transglutaminase is isolated from cultural filtrate of *Strepto verticillium spp., Strepto verticillium mobarens, Strepto verticillium ladakanum, and Strepto verticillium lydicus* Intracellular transglutaminase is secreted by common microbial species *Bacillus subtilis and spherules.*

Biotechnology and Industrial Application of Enzymes:

- Food Processing - Amylases enzymes from fungi and plants are used in production of sugars from starch in making corn-syrup.
- Catalyze enzyme is used in breakdown of starch into sugar, and in baking fermentation process of yeast raises the dough.
- Proteases enzyme help in manufacture of biscuits in lowering the protein level.
- Baby foods - Trypsin enzyme is used in pre-digestion of baby foods.
- Brewing industry - Enzymes from barley are widely used in brewing industries.
- Amylases, glucanases, proteases, betaglucanases, arabinoxylases, amyloglucosidase, are used in prodcution of beer industries.
- Fruit juices - Enzymes like cellulases, pectinases help are used in clarifying fruit juices.
- Dairy Industry - Renin is used inmanufacture of cheese. Lipases are used in ripening blue-mold cheese. Lactases breaks down lactose to glucose and galactose.
- Meat Tenderizes - Papain is used to soften meat.
- Starch Industry - Amylases, amyloglucosidases and glycoamylases converts starch into glucose and syrups.
- Glucose isomerases - production enhanced sweetening properties and lowering calorific values.
- Paper industry - Enzymes like amylases, xylanases, cellulases and liginases lower the viscosity, and removes lignin to soften paper.
- Biofuel Industry - Enzymes like cellulases are used in breakdown of cellulose into sugars which can be fermented.
- Biological detergent - proteases, amylases, lipases, cellulases, asist in removal of protein stains, oily stains and acts as fabric conditioners.

Rubber Industry - Catalase enzyme converts latex into foam.

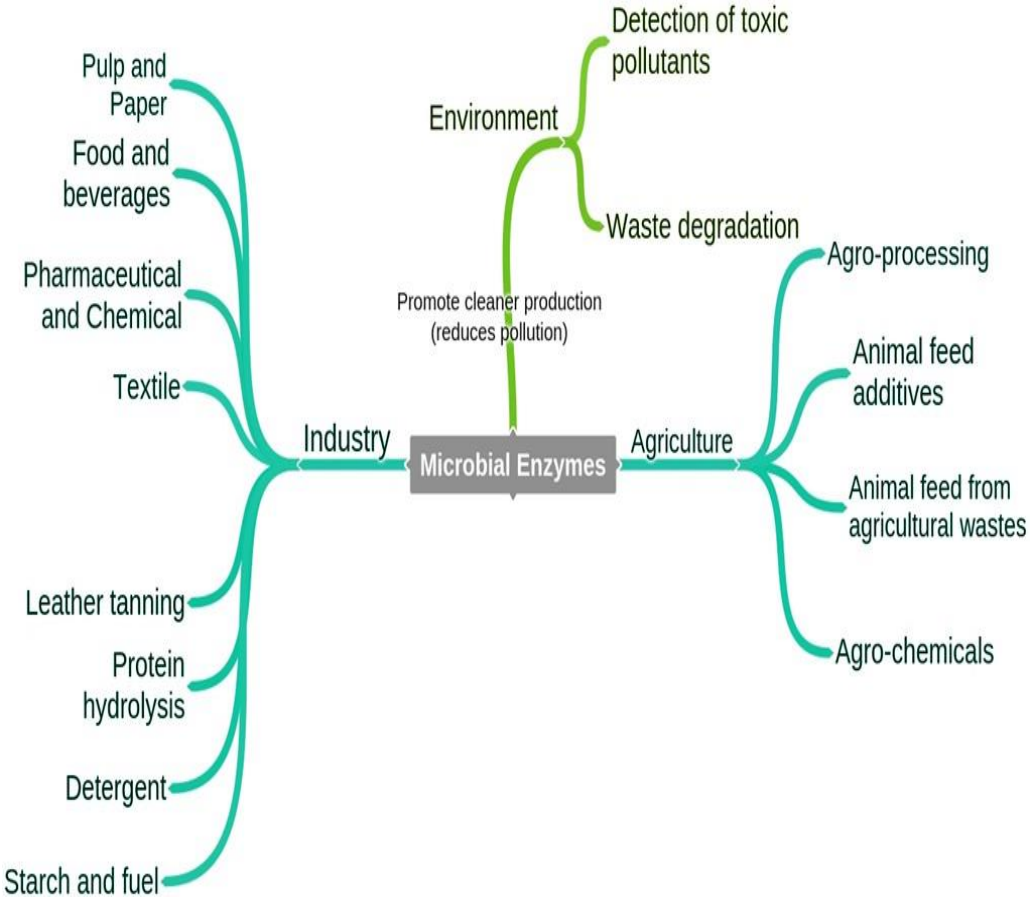
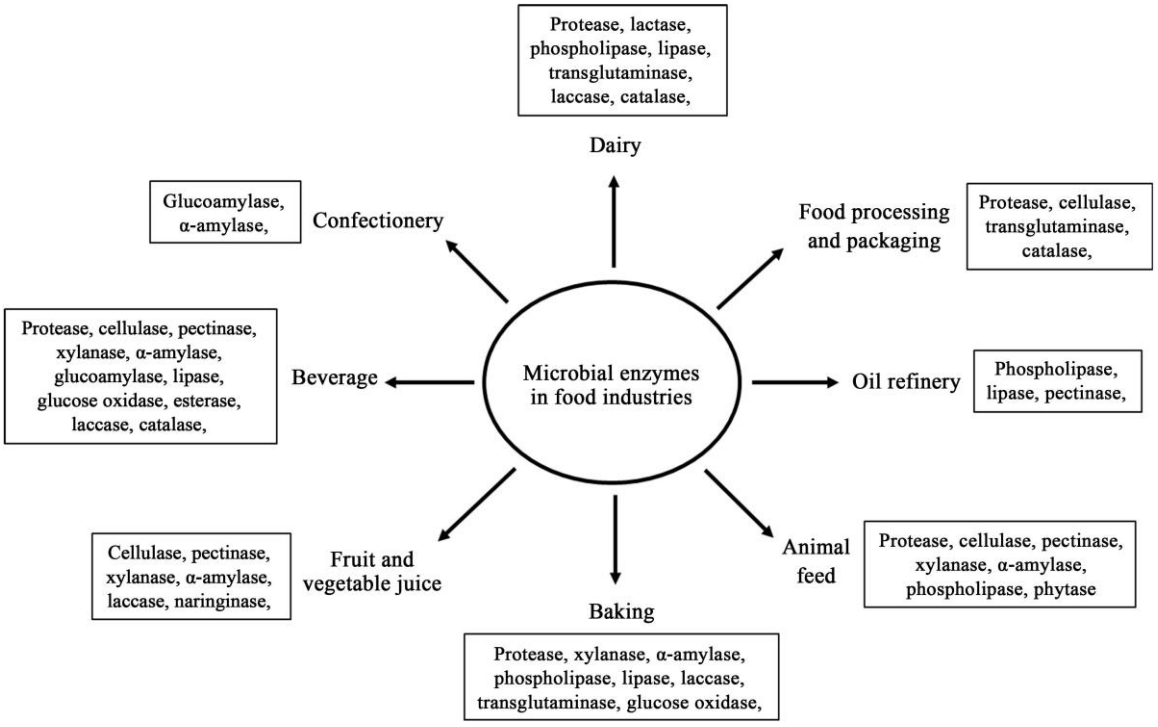


Fig. 1: Applications of Microbial enzymes.

Application of enzymes in food processing

Enzymes are derived from natural sources and may be readily inactivated after a desired transformation has taken place. Unlike inorganic catalysts, enzymes are highly specific, catalyzing the transformation of only a single substrate or the splitting of a small group of closely related compounds or a specific bond. This minimizes by product formation in large-volume reactions. The capacity of enzymes to react under mild conditions of temperature and pH (up to 100°C and pH 3 to 10) achieves a reduction in energy costs. Low usage levels make enzymes economical and practical for commercial application. Because they are derived from plants, animals, or microbial sources, enzymes are perceived as natural, nontoxic food components and are preferred over chemical aids as food-processing aids by consumers. Based on these properties, enzymes find numerous applications in industry .



Proteases

In dairy industry, proteases are added to milk to hydrolyse a peptide bond in kappa-casein, thereby destabilizing casein micelles during the production of curds for cheese-making. Proteases also improve the organoleptic and rheological characteristics of cheese and also reduce the allergenic properties of some fermented milk products. Recently, milk-clotting protease was produced by Mamo *et al.* (2020) from *Aspergillus oryzae* and they showed that the protease from the *Aspergillus oryzae* strain improved the overall organoleptic properties of cheese .

In baking industry, proteases are added to flour during biscuits/crackers production to weaken the gluten protein structure in the flour otherwise the dough will be difficult to handle. The use of protease to weaken the gluten does not affect other nutritional constituents of the dough unlike when a chemical compound, like sodium bisulphite, is used to achieve the same purpose. Consequently, the nutritive value of the biscuits/crackers is improved. Deng *et al.* (2016) reported that acid protease from *Aspergillus usamii* improved the rheological properties of wheat gluten used for baking. Proteases are used in food processing industry to improve the nutritional and functional properties of food materials that are rich in proteins. For instance, in meat processing companies, proteases are used to enhance meat tenderization thereby improving the rheological properties of the meat.

Proteases can be added to animal feed to break down the protein content of the feed into amino acids thereby reducing the anti-nutrient content of the feed.

Cellulases

They hydrolyse cellulases have found some applications in fruit and vegetable juice industry. During the production of fruit and vegetable juice, floating cellulose and hemicellulose from the fruits and vegetables tend to form some

cloudiness in the fruit and vegetable juice. This negatively affects the quality of the fruit and vegetable juice, making the juice less appealing. Consequently, cellulases are used in fruit juice industry as fruit-softening enzymes to hydrolyse cellulose and hemicellulose in raw fruit and vegetable juice thereby enhancing juice extraction, clarification, stabilization, and overall yield

During the processing of fruit and/or vegetable purees, cellulases are used to enhance the yield of purees, minimize damage due to heat and also to reduce the viscosity of purees. Peach, mango, pear, plum, guava, and pawpaw are some of the fruits whose purees have been treated with cellulase during their processing. Animal feed for ruminant animals is usually rich in complex polysaccharides like cellulose, pectin, lignin, and hemicellulose which must be digested by the animal. To improve the digestibility of these animal feed, cellulases are used in processing ruminant animal feed as they can break down cellulose and hemicellulose in animal feed to a more digestible form.

Although cellulase can be produced by a few metazoans like snails, termites, and earthworms, however bacteria and fungi remain the best sources for the production of cellulases for industrial application. Cellulases for food industry application can be produced from fungi such as *Aspergillus niger* .

Pectinases

Pectinases are used in fruit juice industry to degrade pectin (depectinization) in the cell wall of fruits thereby enhancing juice extraction, flavour, clarification, filterability, and overall yield of the fruit juice .In beverage industry, pectinases can be used during the curing stage of wet processing for coffee production as this improves the yield, aroma and flavour of the coffee and also reduces the processing time. Pectinases can also be used in cocoa processing companies to ensure the complete removal of mucilaginous layers of cocoa beans thereby improving the quality of the coffee produced For tea production, treatment of tea leaves with

pectinases reduce the pectin content in the leaves thereby ensuring a relatively quicker fermentation process.

Pectinases are used in animal feed industry to treat animal feed rich in pectin and improve their digestibility for the animals. For oil refinery, pectinases (and some other enzymes which degrade cellulose and hemicellulose) can be added to unprocessed olive oil, coconut oil, sunflower oil, and canola to improve the yield of the refined vegetable oil. Pectinases are ubiquitous but the production of pectinases for industrial applications is mostly achieved through the use of microbes. Pectinases are mostly produced by fungi *Aspergillus niger*, *Penicillium spp* and *Fusarium*.

Lactase

The importance of lactase in the dairy/food and pharmaceutical industries cannot be overemphasized. In dairy industry, lactase is usually added to milk and/or milk-based products (such as whey) to improve their digestibility, especially for lactose-intolerant individuals thereby preventing diarrhoea and tissue dehydration. Also, lactase is used to hydrolyse lactose in ice cream to improve the creamy nature of ice creams and their digestibility. Lactase is used during the production of some milk products to prevent crystallization of lactose thereby making the milk product more soluble.Fungal sources for industrial lactase producing include *Aspergillus oryazae*.

Lipase

The applications of lipase cut across a wide variety of industry including food, detergent/laundry, leather, textile, paper, and pulp processing industries. During cheese production, lipase is used to hydrolyse milk fat into free fatty acids which gives a unique flavour to the cheese, improves its taste, promotes the ripening cheese, and enhances its texture .Lipase is used in wine production and beverage-making companies to improve the aroma of wine and beverage, respectively. Many products made in baking industry require the use of egg white as an ingredient. Egg white contains about 0.02% of lipid which is sufficient to decrease

the quality of the dough Lipase can be used to catalyse the hydrolysis of the lipid content of egg white thereby improving the quality of the baked product.

Glucose Oxidase (EC 1.1.3.4)

Glucose Oxidase (EC 1.1.3.4) is an oxidoreductase that catalyses the oxidation of glucose to D-glucono-*δ*-lactone and hydrogen peroxide. In food processing industry, glucose oxidase is added to glucose-containing food substances to produce the food additive, D-glucono-*δ*-lactone. Once produced, D-glucono-*δ*-lactone acts as a preservative to enhance the quality, flavour, and stability of the food. The oxidizing property of glucose oxidase is employed in baking industry to produce stronger dough. Glucose oxidase can also be used to increase the vol ume of bread during baking. In a recent investigation by Ge *et al.*, (2020), they produced a novel glucose oxidase and showed that the enzyme significantly in- creased the volume of bread during baking. Glucose oxidase is added to diabetic drinks to reduce the glucose content of the drinks thus making them favourable for consumption by diabetics. Fungi are the most common microbial source for the production of glucose oxidase on an industrial scale. Examples of glucose oxidase-producing fungi include *Aspergillus niger Mucor circinelloides, Penicillium glaucum*, so serve as a preservative in some baking products

Laccase (EC 1.10.3.2)

Laccase (EC 1.10.3.2) is a multi-copper oxidase that catalyses the oxidation of phenolic compounds, aromatic amines, and ascorbic acid. Laccase is produced by fungi, bacteria, soil algae and some insects. However, the laccase produced by these organisms tends to perform different functions. For example, laccase from microbial sources (bacteria and fungi) catalyses the degradation of lignin in wood to release hemicellulose and cellulose components of the wood while lac- case from plant sources tend to catalyse the biosynthesis of lignin in plants. Laccase is used in wine

industry for wine stabilization. Laccase has also been used to remove oxygen from wine to increase its shelf life.

Laccase is also used in beverage-producing companies to modify the colour of beverages.In brewing industry, laccase is used to minimize the formation of haze through the oxidation of polyphenols during beer production Additionally, laccase is used to remove oxygen during beer production, thus acting as a preservative.

The oxidizing property of laccase is applied in baking industry as it makes the baking process much better. In fruit juice industry, laccase can be used alone or with cellulase and pectinase for improved clarification and overall yield of fruit juice. Laccase can be used in dairy industry to treat skim milk yoghurt to improve the quality of the yoghurt by crosslinking milk proteins with laccase .Fungi are one of the best sources for the production of laccase for industrial applications. Some laccase producing fungi include *Trametes versicolor*, *Pleurotus eryngii*, *Funalia trogii*,

Catalase (EC 1.11.1.6)

Catalase (EC 1.11.1.6) is an enzyme that catalyses the reduction of hydrogen peroxide to water and oxygen. Catalase is found in every aerobic living organism that is exposed to oxygen. Consequently, it can be produced by plants, animals, and microbes. Like laccase, catalase is used in wine-making companies to remove oxygen from wine to further elongate its shelf life and also for reducing alcohol in wines. In dairy industry, catalase is used to remove peroxide from milk products to prevent milk rancidity. Oxidation of food items inside food wrappers is one of the causes of food spoilage. Catalase is used during food packaging to prevent oxidation of food items thereby elongating their shelf life.The production of catalase for industrial application are mostly from microbial sources. Some identified microbial sources of catalase for industrial applica- tion are *Aspergillus niger*, *Micrococcus luteus*, *Ba-cillus maroccanus*, *Pyrobaculum calidifontis*, *Rhizobium radiobacter*.

Phytase (EC 3.1.3.8)

Phytase (EC 3.1.3.8) is a kind of phosphatase that catalyses the hydrolytic release of inorganic phosphorus from phytic acid contained in grains and oilseeds. The enzymatic activity of phytase enhances the uptake of minerals like calcium, zinc, and iron which are sometimes bound to phytic acid in the grains and oilseeds. Thus, the hydrolytic activity of phytase on phytic acid increases the bioavailability of calcium, zinc, and iron to monogastric animals. Phytase is a natural enzyme that is found in fungi, bacteria, plants, and ruminant animals. Phytase is added to the feed of monogastric animals to improve the nutritive value of animal feed by enhancing the uptake of important minerals bound to phytic acid in their feed. Fungi and bacteria are the predominant sources for the industrial production of phytase. Microbial sources of phytase include *Aspergillus niger*, *Aspergillus fumigatus*, *Escherichia coli*, *Bacillus amyloliquefaciens*, *Bacillus subtilis.*

General Therapeutic Application of Other Enzymes

Therapeutic enzymes have a wide variety of specific uses such as oncolytics, thrombolytics, or anticoagulants and as replacements for metabolic deficiencies. Proteolytic enzymes serve as good anti-inflammatory agents. The list of enzymes which have the potential to become important therapeutic agents and its microbial sources. A number of factors severely decrease the potential utility of microbial enzymes once we enter the medical field due to large molecular size of biological catalyst which prevents their distribution within somatic cells, and another reason is the response of immune system of the host cell after injecting the foreign enzyme protein.

a-Treatment of Damaged Tissue

A large number of proteolytic enzymes of plant and bacterial origin have been studied for the removal of dead skin of burns. Various enzymes of higher quality and purity are now in clinical trials. Debrase gel dressing, containing a mixture of several enzymes extracted from pineapple, received clearance in 2002 from the US FDA for a Phase II clinical trial for the treatment of partial-thickness and full-thickness burns. A proteolytic enzyme (VibrilaseTM) obtained from *Vibrio proteolyticus* is found to be effective against denatured proteins such as those found in burned skin.

b-Treatment of Infectious Diseases

Lysozyme is a naturally occurring antibacterial agent and used in many foods and consumer products, as it is able to breakdown carbohydrate chains in bacterial cell wall. Lysozyme has also been found to have activity against HIV, as RNase A and urinary RNase U present selectively degrade viral RNA showing possibilities for the treatment of HIV infection. Chitinases is another naturally occurring antimicrobial agent. The cell wall of various pathogenic organisms, including fungi and protozoa is made up of chitin and is a good target for antimicrobials. The lytic enzyme derived from bacteriophage is used to target the cell walls of *Streptococcus pneumonia, Bacillus anthracis,* and *Clostridium perfringens* .The application of lytic bacteriophages can be used for the treatment of several infections and could be useful against new drug-resistant bacterial strains.

c- Treatment of Cancer

The cancer research has some good instances of the use of enzyme therapeutics. Recent studies have proved that arginine-degrading enzyme (PEGylated arginine deaminase) can inhibit human melanoma and hepatocellular carcinomas.

Currently, another PEGylated enzyme, Oncaspar (pegaspargase), has shown

good results for the treatment of children newly diagnosed with acute lymphoblastic leukemia and are already in use in the clinic.

The normal cells are able to synthesize asparagine but the cancerous cells cannot and thus, die in the presence of asparagine degrading enzyme. The further application of enzymes as therapeutic agents in cancer is described by antibody-directed enzyme prodrug therapy (ADEPT). A monoclonal antibody carries an enzyme specific to cancer cells where the enzyme activates a prodrug and destroys cancer cells but not normal cells. This approach is being utilized for the discovery and development of cancer therapeutics based on tumor-targeted enzymes that activate prodrugs. The targeted enzyme prodrug therapy (TEPT) platform, involving enzymes with antibody-like targeting domains, will also be used in this effort.

Enzymes in paper and pulp industry

The paper and pulp industry requires a step of separation and degradation of lignin from plant material, where the pretreatment of wood pulp using ligninolytic enzymes is important for a milder and cleaner strategy of lignin removal compared to chemical bleaching. Bleach enhancement of mixed wood pulp has been achieved using co-culture strategies, through the combined activity of xylanase and laccase. Fungi are the most potent producers of lignin degrading enzymes.

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