2. REVIEW

2.1 ENZYMES ARE PROTEIN CATALYSTS

A catalyst is a molecule that increases the rate of a chemical reaction without being consumed by the reaction. When the reaction is finished, the amount of catalyst does not change. An enzyme is a substance produced by a living organism which acts as a catalyst to bring about a specific biochemical reaction. They are proteins produced in tiny quantities by all living organisms (bacteria, plants and animals) and functioning as highly selective biochemical catalysts in converting one molecule into another. The key reactions in cell metabolism are catalyzed by enzymes, which allow some desired reactions to happen quickly while not changing the rates of other reactions (Dixon and Webb, 1964).

Chemists have known for almost a century that for most chemical reactions to proceed, some form of energy is needed. In chemistry, activation energy is a term introduced in 1889 by the Swedish scientist Svante Arrhenius to describe the minimum energy which must be available to a chemical system with potential reactants to result in a chemical reaction. It is the magnitude of the activation energy which determines just how fast there action will proceed. Similarly in the biological world, enzymes are the catalysts which lower the activation energy for the reaction they are catalyzing. The enzyme is thought to reduce the "path" of the reaction. This shortened path would require less energy for each molecule of substrate converted to product. The lower the activation energy, the faster a reaction happens. Given a total amount of available energy, more molecules of substrate would be converted when the enzyme is present (the shortened "path") than when it is absent. Hence the reaction is said to go faster in a given period of time.
2.2 ACTION OF ENZYME

In order for a reaction to take place, some or all of the chemical bonds in the reactants must be broken so that new bonds, those of the products, can form. To get the bonds into a state that allows them to break, the molecule must be contorted (deformed or bent) into an unstable state called the transition state. The transition state is a high-energy state, and some amount of energy, the activation energy must be added in order for the molecules to reach it. Because the transition state is unstable, reactant molecules do not stay there long, but quickly proceed to the next step of the chemical reaction.

The source of activation energy is typically heat, with reactant molecules absorbing thermal energy from their surroundings. This thermal energy speeds up the motion of the reactant molecules, increasing the frequency and force of their collisions, and also jostles the atoms and bonds within the individual molecules, making it more likely that bonds will break. Specifically, the higher the activation energy, the slower the chemical reaction will be. This is because molecules can only complete the reaction once they have reached the top of the activation energy barrier. The process of speeding up a reaction by reducing its activation energy is known as catalysis, and the factor that is added to lower the activation energy is called a catalyst. Biological catalysts are known as enzymes that help to lower the activation energy of a reaction, and to thereby increase reaction rate.

For example in the Haber process for ammonia synthesis, elevated temperatures (500°C) and pressure of several hundred atmospheres and iron based catalyst are used. The Haber 16 process requires high pressures (around 200 atm) and high temperatures (at least 400°C), routine conditions for industrial catalysis. On the contrary the nitrogen fixing bacteria can convert the inert dinitrogen molecule into ammonia at ordinary temperature and pressure in the presence of an enzyme complex nitrogenase, which has iron and molybdenum for the activity.
2.3 FACTORS INFLUENCING ENZYME REACTION

Five major parameters are known to affect an enzyme catalyzed reaction, which are as follows:

**Enzyme Concentration**

The effect of enzyme concentration on the rate of reaction is shown in Fig.3. At low enzyme concentrations there are more substrate molecules than there are available active sites. Increasing the number of active sites by increasing the concentration of the enzyme, therefore, effectively increases the rate of the reaction. Eventually, at point x, increasing the enzyme concentration has no effect on the rate of reaction. This is because it is now the number of substrate molecules which has become the limiting factor.

**Substrate concentration**

At low substrate concentration the reaction proceeds slowly. This is because there are not enough substrate molecules to occupy all of the active sites on the enzyme. As substrate concentration increases, the rate increases because there are
more enzyme substrate complexes formed. At a particular stage, however, increasing the substrate concentration will have no further effect on the rate of reaction. This is because all of the enzyme’s active sites are now occupied by substrate molecules – increasing the substrate concentration further will have no effect, because no more enzyme substrate complexes can form. The rate of reaction now depends on the turnover rate of the enzyme, i.e. the number of substrate molecules transformed by one molecule of enzyme per second.

**Enzyme Inhibitors**

Inhibitors slow down the rate of reaction. As such, they are an essential form of cellular control, allowing enzyme reaction rate to be slowed when necessary. Some enzymes are inhibited by the end product of the reaction they catalyse.

(a) **Reversible inhibitors**

There are two types of reversible inhibitor:

- competitive reversible inhibitor
- non-competitive reversible inhibitor

(b) **Irreversible inhibitors**

Irreversible inhibitors bind covalently and permanently to the enzyme, preventing normal enzyme function. For example, aspirin is an irreversible inhibitor of cyclooxygenase, an enzyme involved in the synthesis of prostaglandins. Substances such as mercury, iron and arsenic bind irreversibly to the SH (sulphydryl) group on enzymes.

**Temperature**

Enzymes have an optimum temperature - this is the temperature at which they work most rapidly. Below the optimum temperature, increasing temperature will increase the rate of the reaction. This is because temperature increases the kinetic
energy of the system, effectively increasing the number of collisions between the substrate and the enzyme’s active site. Temperatures above the optimum will lead to denaturation. This occurs because the hydrogen bonds and disulphide bridges which maintain the shape of the active site are broken. Thus, enzyme substrate complexes can no longer be formed. The effect of temperature on the rate of a chemical reaction is described by the term “temperature coefficient” (Q10).

\[
Q_{10} = \frac{\text{rate of reaction at } T + 10^\circ\text{C}}{\text{rate of reaction at } T^\circ\text{C}}
\]

Many enzymes have a Q10 of between 2 and 3. In other words, provided that the temperature is not so high that it causes denaturation, an increase in temperature of 10°C will speed up the reaction by a factor of 2-3, that is it will double or treble it.

**pH**

The effect of a change in pH on enzyme activity is shown in Fig 3. As with temperature, each enzyme has an optimum pH. If pH increases or decreases much beyond this optimum, the ionisation of groups at the active site and on the substrate may change, effectively slowing or preventing the formation of the enzyme substrate complex. At extreme pH, the bonds which maintain the tertiary structure, the active site, are disrupted and the enzyme is irreversibly denatured.

In addition to temperature and pH, there are other factors, such as ionic strength, which can affect the enzymatic reaction.
Fig. 3: Factors influencing enzyme reaction
(Enzyme and Substrate Concentration, Temperature and pH)

2.4 ENZYME PURIFICATION

Purification of enzymes dates back to 1922 and the first crystalline enzyme urease was purified by Sumner in 1926. The main part of purification consists of a series of fractionations by which the enzyme protein is separated from the other proteins present. Fractional precipitation is done by a combination of the following methods like; change of pH, denaturation by heating, with organic solvents or with salts or by adsorption and finally crystallization is done to obtain a pure enzyme (Berg et al., 2002).

For many industrial applications, partially purified enzyme preparations will suffice. However, enzymes for analytical purposes and for medical use must be highly
purified. Special procedures employed for enzyme purification are crystallization, electrophoresis, and chromatography. However crystallization and electrophoresis are not relevant for large scale purifications. Chromatography, in contrast, is of fundamental importance to enzyme purification. Molecules are separated according to their physical properties (size, shape, charge, hydrophobic interactions), chemical properties (covalent binding), or biological properties (biospecific affinity). Modern industries apply ultra filtration to produce enzymes.

Since the current work is directed towards commercial production, two major relevant protein purification methods are highlighted here. They are as follows:

2.4.1 Separation by salt precipitation

Proteins are usually poorly soluble in pure water. The most common type of precipitation for proteins is salt-induced precipitation. At low concentration of the salt, solubility of the proteins usually increases slightly (salting in). But at high concentrations of salt, the solubility of the proteins decreases sharply (salting out) and the proteins precipitate out.

2.4.2 Separation by ultrafiltration

Ultrafiltration (UF) is a membrane filtration technique which enables concentration, fractionation and purification of macromolecules in solution to be carried out at temperatures close to ambient, and without phase change or addition of chemicals or solvents. This protects the product's biochemical structure and activity, giving increased yields over conventional technologies while simplifying total processing schemes. The reduction of large volumes to manageable amounts and production of the required purification standard is the efficiency of the technique that makes it a reliable one in the biotechnology industries (Short and Webster, 1992).
The advantages of ultrafiltration can be summarized as follows:

- UF fulfills the dual requirement of concentration and purification simultaneously.
- UF involves no phase change, heat treatment or addition of chemicals.
- UF reduces the risk of bacterial contamination and simplifies automated cleaning especially in the case of self-supporting hollow fibers.
- Low pressure ultrafiltration systems which operate in the laminar or transition flow regions do not cause shear damage to retentates.
- Full draining of UF systems and mild processing conditions increase overall product yield.
- pH and ionic strength are not altered during UF processing.
- UF is substantially less expensive than evaporation or freeze drying.

2.5 PURITY CHECK FOR PROTEIN

2.5.1 SDS-PAGE

Prior to any downstream experiment, purity and integrity are the very first qualities that need to be assessed for any protein sample. This is routinely achieved by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS–PAGE). This technique, associated with Coomassie blue staining, can detect bands containing as little as 100 nano gram of protein in a simple and relatively rapid manner (just a few hours) (Raynal et al., 2014).

Polyacrylamide gel electrophoresis (PAGE), describes a technique widely used in biochemistry, to separate biological macromolecules, usually proteins or nucleic acids, according to their electrophoretic mobility. Mobility is a function of the length, conformation and charge of the molecule. For proteins, sodium dodecyl sulfate (SDS) is an anionic detergent applied to protein samples to linearize proteins and to
impart a negative charge to linearized proteins. This procedure is called SDS-PAGE. In most proteins, the binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis.

2.5.2 Spectral analysis

Spectroscopy is a technique that measures the interaction of molecules with electromagnetic radiation. Absorption spectroscopy is usually performed with molecules dissolved in a transparent solvent, such as aqueous buffers. The absorbance of a solute depends linearly on its concentration and therefore absorption spectroscopy is ideally suited for quantitative measurements (Schmid, 2001). www.els.net.(12).

Principle

Proteins in solution absorb ultraviolet light with absorbance maxima at 280 and 200 nm. Amino acids with aromatic rings are the primary reason for the absorbance peak at 280 nm. Peptide bonds are primarily responsible for the peak at 200 nm.

Analysis

1. *Unknown proteins or protein mixtures.* Path length for most spectrometers is 1 cm. The following formula is used to roughly estimate protein concentration.

   Concentration (mg/ml) = Absorbance at 280 nm divided by path length (cm).

2. *Unknowns with possible nucleic acid contamination.* the following formula to estimate protein concentration:

   Concentration (mg/ml) = (1.55 x A280) - 0.76 x A260)
When measuring purified proteins the 260/280 ratio can be a useful tool to determine the purity of your isolated protein. An ideal 260/280 ratio for common proteins is 0.6. Higher ratios may indicate contamination of isolated proteins. http://www.ruf.rice.edu/~bioslabs/methods/protein/abs280.html (13).

2.5.3 Activity of the isolated protein

Once the homogeneity of the protein of interest has been assessed, one has to ensure it is active and functional. An infinite variety of generic or protein-specific functional assays has been designed, relying principally on catalytic and binding properties. A well established general protease assay is widely applied to find out the active nature of the protein of interest (Kunitz, 1947).

2.6 ENZYMES IN THE INDUSTRY

Enzymes are used in the chemical industry and other industrial applications when extremely specific catalysts are required. Though there are thousands of enzyme, proteases are the most widely used enzymes throughout the world “Enzymes Used in Industry” (2016). https://www.boundless.com/microbiology/textbooks/boundless-microbiology-text-book/industrial-microbiology-17/microbial-products-in-the-health-industry-199/enzymes-used-in-industry-1004-5469/(14). Due to the present energy crisis and the awareness for energy conservation, it is desirable to use proteases that are active at lower temperatures.

2.6.1 Proteases Are Common and Widespread Enzymes

A protease (also called a peptidase or proteinase) is any enzyme that performs proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in a polypeptide chain. Analysis of complete genomes has shown that about 2% of proteins in all kinds of organisms are proteases.
Proteases have evolved multiple times, and different classes of protease can perform the same reaction by completely different catalytic mechanisms. Proteases occur in all organisms, from prokaryotes to eukaryotes to viruses. These enzymes are involved in a multitude of physiological reactions from simple digestion of food proteins to highly regulated cascades (e.g., the blood-clotting cascade, the complement system, apoptosis pathways, and the invertebrate prophenoloxidase-activating cascade), processing of proteins, protein turnover, cell division, metabolism, etc. Proteases can either break specific peptide bonds (limited proteolysis), depending on the amino acid sequence of a protein, or break down a complete peptide to amino acids (unlimited proteolysis). The activity can be a destructive change (abolishing a protein's function or digesting it to its principal components), it can be an activation of a function, or it can be a signal in a signaling pathway.

The field of protease research is enormous. In 2004, approximately 8000 papers related to this field were published each year (Barret et al., 2012).

2.6.2 Proteases can be classified into seven broad groups:

1. Serine proteases - using a serine alcohol
2. Cysteine proteases - using a cysteine thiol
3. Threonine proteases - using a threonine secondary alcohol
4. Aspartic proteases - using an a spartate carboxylic acid
5. Glutamic proteases - using a glutamate carboxylic acid
6. Metalloproteases - using a metal, usually zinc
7. Asparagine peptide lyases - using an as paragine to perform an elimination reaction (not requiring water).
2.6.3 Mechanisms of Protease

Polypeptides can be cleaved either chemically or enzymatically. Enzymes that catalyse the hydrolytic cleavage of peptide bonds are called proteases. Proteases fall into four main mechanistic classes: serine, cysteine, aspartyl and metalloproteases. In the active sites of serine and cysteine proteases, the eponymous residue is usually paired with a proton-withdrawing group to promote nucleophilic attack on the peptide bond. Aspartyl proteases and metalloproteases activate a water molecule to serve as the nucleophile, rather than using a functional group of the enzyme itself. However, the overall process of peptide bond scission is essentially the same for all protease classes. Soluble serine proteases (a); cysteine proteases (b); aspartyl proteases (c); and metalloproteases (d) (Erez et al., 2009).

![Fig.4: Mechanism of Protease](image)

2.6.4 Plant Proteases

Though there are four types of plant enzymes, namely proteases, amylases, lipases and cellulases the interest received by proteases has increased significantly. They are widely used in medicine and the food industry. Some proteases, like papain,
bromelain and ficin are used in various processes such as brewing, meat softening, milk-clotting, cancer treatment, digestion and viral disorders. Proteases are used in industry, medicine and as a basic biological research tool (Hooper, 2002). These enzymes can be obtained from their natural source or through in vitro cultures, in order to ensure a continuous source of plant enzymes (González-Rábadea, 2011).

Digestive proteases are part of many laundry detergents and are also used extensively in the bread industry in bread improver. A variety of proteases are used medically both for their native function (e.g. controlling blood clotting) or for completely artificial functions (e.g. for the targeted degradation of pathogenic proteins). Highly specific proteases such as TEV protease and thrombin are commonly used to cleave fusion proteins and affinity tags in a controlled fashion. https://en.wikipedia.org/wiki/Protease, 20 June 2016 (15).

2.6.5 Cysteine proteases

The enzyme of the current research falls under the cysteine category. Cysteine proteases, also known as thiol proteases, are enzymes that degrade proteins. These proteases share a common catalytic mechanism that involves a nucleophilic cysteine thiol in a catalytic triad or dyad.

Cysteine proteases are commonly encountered in fruits including the papaya, pineapple, fig and kiwi fruit. {Papain (Carica papaya), bromelain (Ananas comosus), cathepsin K (liverwort)}. The proportion of protease tends to be higher when the fruit is unripe. In fact, dozens of latices of different plant families are known to contain cysteine proteases (Domsalla and Melzig, 2008). Cysteine proteases are used as an ingredient in meat tenderizers. Cysteine proteases are used as feed additives for livestock to improve the digestibility of protein. They hydrolyse complex proteins into simple amino acids through gut. Recent advances in the industries explicitly prove the use of papain from tanneries to breweries and pharmaceutical to cosmetics. As a
highlight of its significance it is used in molecular biology for synthesis of biomolecules, as an antibody purification tool in immunology, as a marker gene and the list never seems to fade.

2.7 CHARACTERISTICS OF PAPAIN

Papain is a cysteine protease of the peptidase C1 family. Papain consists of a single polypeptide chain with three disulfide bridges and a sulphydryl group necessary for activity of the enzyme.

Molecular weight: 23,406 Da (amino acid sequence)

Optimal pH for activity: 6.0-7.0

Temperature Optimum for Activity: 65 °C

pI: 8.75; 9.55

Spectral properties:

\( \lambda_{\text{max}}: 278 \text{ nm} \)


2.7.1 The Complete Amino Acid Sequence of Papain

The papain precursor protein contains 345 amino acid residues, and consists of a signal sequence (1-18), a propeptide (19-133) and the mature peptide (134-345). The amino acid numbers on papain are based on the mature peptide. The protein is stabilized by the three disulfide bridges. Its three-dimensional structure consists of two distinct structural domains with a cleft between them (15, 16, 17). The active site, consisting of a cysteine and a histidine, lies at the surface of the cleft. Apart from four
short alpha helical segments and one short beta structure the conformation of the chain is irregular (Paul et al., 2013).

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>No.</th>
<th>Amino Acid</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>10</td>
<td>Glycine</td>
<td>28</td>
</tr>
<tr>
<td>Histidine</td>
<td>2</td>
<td>Alanine</td>
<td>14</td>
</tr>
<tr>
<td>Arginine</td>
<td>12</td>
<td>Valine</td>
<td>18</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>7</td>
<td>Isoleucine</td>
<td>12</td>
</tr>
<tr>
<td>Asparagine</td>
<td>12</td>
<td>Leusine</td>
<td>11</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>8</td>
<td>Tyrosine</td>
<td>19</td>
</tr>
<tr>
<td>Glutamine</td>
<td>12</td>
<td>Phenylalanine</td>
<td>4</td>
</tr>
<tr>
<td>Threonine</td>
<td>8</td>
<td>Tryptophan</td>
<td>5</td>
</tr>
<tr>
<td>Serine</td>
<td>13</td>
<td>Cysteine</td>
<td>1</td>
</tr>
<tr>
<td>Proline</td>
<td>10</td>
<td>Half Cysteine</td>
<td>6</td>
</tr>
</tbody>
</table>

![Fig.5: Complete sequence of papain enzyme](image-url)
2.8 APPLICATION OF PROTEASES

Proteases have a large variety of applications, mainly in the detergent and food industries. In view of the recent trend of developing environment friendly technologies, proteases are envisaged to have extensive applications in leather treatment and in several bioremediation processes. The worldwide requirement for enzymes for individual applications varies considerably. Proteases are used extensively in the pharmaceutical industry for preparation of medicines such as ointments for debridement of wounds, etc. Proteases that are used in the food and detergent industries are prepared in bulk quantities and used as crude preparations, whereas, those that are used in medicine are produced in small amounts but require extensive purification before they can be used.
For brevity sake the varied application of plant proteases and especially papain has been outlined. Since the current research had the tannery in focus a brief account of enzymes in the leather industry is sketched here.

2.8.1 Application of proteases in the Leather Industry

Leather processing involves several steps such as soaking, dehaiering, bating, and tanning. The major building blocks of skin and hair are proteinaceous. The conventional methods of leather processing involve hazardous chemicals such as sodium sulfide, which create problems of pollution and effluent disposal. The use of enzymes as alternatives to chemicals has proved successful in improving leather quality and in reducing environmental pollution. Proteases are used for selective hydrolysis of noncollagenous constituents of the skin and for removal of nonfibrillar proteins such as albumins and globulins. The purpose of soaking is to swell the hide. Traditionally, this step was performed with alkali. Currently, microbial alkaline proteases are used to ensure faster absorption of water and to reduce the time required for soaking. The use of non-ionic and, to some extent, anionic surfactants is compatible with the use of enzymes. The conventional method of dehairing and dewooling consists of development of an extremely alkaline condition followed by treatment with sulfide to solubilize the proteins of the hair root. At present, alkaline proteases with hydrated lime and sodium chloride are used for dehairing, resulting in a significant reduction in the amount of wastewater generated. Earlier methods of bating were based on the use of animal feces as the source of proteases; these methods were unpleasant and unreliable and were replaced by methods involving pancreatic trypsin. Currently, trypsin is used in combination with other *Bacillus* and *Aspergillus* proteases for bating. The selection of the enzyme depends on its specificity for matrix proteins such as elastin and keratin, and the amount of enzyme needed depends on the type of leather (soft or hard) to be produced. Increased usage of enzymes for dehairing and bating not only prevents pollution problems but also is effective in saving energy. Novo Nordisk manufactures three different proteases, Aquaderm, NUE, and Pyrase, for use in soaking, dehaiering, and bating, respectively.
2.9 PAPAYA PRODUCTION

Papayas are produced in about 60 countries, with the bulk of production occurring in developing economies. Global papaya production in 2010 was estimated at 11.22 Mt, growing at an annual rate of 4.35 percent between 2002 and 2010 (global production in 2010 was 7.26% higher than 2009, and 34.82% higher than 2002). Asia has been the leading papaya producing region, accounting for 52.55 percent of the global production between 2008 and 2010, followed by South America (23.09%), Africa (13.16%), Central America (9.56%), the Caribbean (1.38%), North America (0.14%), and Oceania (0.13%) (FAOSTAT 2012a) (Fig.7).

![Fig.7: Global Production of Papaya](image)

2.9.1 Planting Material

Papaya is commercially propagated by seed and tissue culture plants. The seed rate is 250-300 g/ha. The seedlings can be raised in nursery beds 3m long, 1m wide and 10 cm high as well as in pots or polythene bags. The seeds after being treated with 0.1% Monosan (phenyl mercuric acetate),ceresan etc. are sown 1 cm deep in rows 10 cm apart and covered with fine compost or leaf mould. Light irrigation is provided during the morning hours. The nursery beds are covered with polythene
sheets or dry paddy straw to protect the seedlings. About 15-20 cm tall seedlings are chosen for planting in about two months. Papaya is planted during spring (February-March), monsoon (June-July) and autumn (October-November). A spacing of 1.8 x 1.8 m is normally followed. However higher density cultivation with spacing of 1.5 x 1.5 m/ha enhances the returns to the farmer and is recommended. A closer spacing of 1.2 x 1.2 m for cv. Pusha Nanha is adopted for high density planting, accommodating 6,400 plants/ha.

2.9.2 Planting Method

The seedlings are planted in pits of 60x60x60 cm size. In the summer months the pits are dug about a fortnight before planting. The pits are filled with top soil along with 20 kg of farmyard manure 1 kg neem cake and 1 kg bone meal. Tall and vigorous varieties are planted at greater spacing while medium and dwarf ones at closer spacing.

2.9.3 Nutrition

Papaya plant needs heavy doses of manures and fertilizers. Apart from the basal dose of manures (@ 10 kg/plant) applied in the pits, 200-250 g each of N, P₂O₅ and K₂O are recommended for getting high yield. Application of 200 g N is optimum for fruit yield but papain yield increases with increase in N up to 300 g.

2.9.4 Micronutrients

Micro-nutrients viz. ZnSO₄ (0.5%) and H₂BO₃ (0.1%) are sprayed in order to increase growth and yield characters.

2.9.5 Irrigation

The irrigation schedule is fixed on the basis of soil type and weather conditions of the region. Protective irrigation is provided in the first year of planting. During the second year, irrigation is provided at fortnightly interval in winter and at
an interval of 10 days in summer. Basin system of irrigation is mostly followed. In areas having low rainfall, sprinkler or drip system can be adopted. Earthing up is done before or after the onset of monsoon to avoid water-logging and also to help the plants to stand erect.

2.9.6 Removal of male plants

About 10% of the male plants are kept in the orchards for good pollination where dioecious varieties are cultivated. As soon as the plants flower, the extra male plants are uprooted.

2.9.7 Plant Protection Measures

Insect Pests

The insect pests mostly observed are fruit flies (*Bactrocera cucurbitae*), ak grasshopper (*Poekilocerus pictus*), aphids (*Aphis gossypii*), red spider mite (*Tetranychus cinnabarinus*), stem borer (*Dasyses rugosellus*) and grey weevil (*Myllocerus viridans*). In all cases the infected parts need to be destroyed along with application of prophylactic sprays of Dimethoate (0.3%) or methyl demeton (0.05%).

Diseases

The main diseases reported are powdery mildew (*Oidium caricae*), anthracnose (*Colletotrichum gloeosporioides*), damping off and stem rot. Application of wettable sulphur (1g/L) carbendazim / thiophanate methyl (1g/L) and Kavach / Mancozeb (2 g/L) has been found to be effective in controlling the diseases.

2.9.8 Cultivation of papaya for papain

Papaya for papain extraction is a special variety. Seedlings should be prepared in raised beds in sunny areas. The seed requirement for 1 acre is 400g seeds. The seedling is ready in 60 days. The land should be prepared well and seedlings should
be transplanted at a spacing of 6 x 6 feet. The seedlings are planted at a depth of ¾ feet. The seedling requirement of 1 acre is 1500. The most difficult part in papaya cultivation is the identification of male and female plants. Female plants should be more to get high yield. But during planting the plants cannot be differentiated. It takes 5 months for identification. In female trees flowers are borne single. Whereas in male plants flowers appear in bunches. After identification, the population should be maintained in the rate of 20 females: 1 male. The extra male plants should be cut back. Based on weed growth, one or two weeding is done in a year. For the first 9 months only irrigation is done. The ground should not be moist as it can cause rotting in the plants. During the 6th month give basal fertilizers, later once in 6 months apply 300g of mixed fertilizers for each plant.

Tamil Nadu Agriculture University, Coimbatore has developed CO2 and CO5 varieties which are suitable for obtaining good latex production. Generally, papain manufacturers grow pawpaw to cater to their needs or else they could collect from nearby farmers. For obtaining 50-60 kg of latex per day for 300 days there is need for cultivation on 25 acres of land. The land is divided into 3 sections the plantation is done in one section at a time in a cycle of 3-4 months so as to make availability of latex round the year.

2.10 PAPAYA LATEX

2.10.1 Collection of latex

Papaya milk can be extracted from the 9th month to the 18th month. White milky latex of green and fully grown papaya fruits is collected in the early morning by making deep longitudinal cuts by stainless steel or wooden sharp knives. Latex is collected in stainless steel trays while latex coagulated in the surface of the fruits is scrapped and collected in the trays or rexin sheets. The milk becomes wax like in a short time. It is scooped and packed in plastic bags. A fruit is tapped about 6 times in
the course of 16 days. This latex is passed through 50 mesh sieves to remove dirt and then it is mixed with potassium meta bisulphate.

This is sent to the cold storage unit from where the company procures it. In 2 ½ years, 1 acre gives 1700 kg of papain, 100 tonnes of unripe fruit or 50 tonnes of ripe fruit.

Several thousand metric tons of papaya latex are obtained each year from the unripe fruits. An orchard of not less than 10 hectares is usually required to produce one metric ton of dry latex annually (El Moussaoui et al., 2001).

2.10.2 Bioactive moieties in the latex

The latex of the tropical species Carica papaya is well known for being a rich source of the four cysteine endopeptidases i.e., papain (less than 10%), chymopapain A and B (26–30%), glycyl endopeptidase III and IV (23–28%), and caricain (14–26%). These form 69–89% of its total protein content. Broklehurst and Salih (1983) published a rational basis for the identification of the proteases in the papaya is as follows: Major cysteine proteinase components given in order of increasing basicity: papain (papaya peptidase I; 1 SH/molecule), chymopapain A (2 SH/molecule), chymopapains B1-B3 (2 SH/molecule) and papaya peptidase II (1 SH/molecule). Altogether, these enzymes are present in the laticifers at a concentration higher than 1 mM. The proteinases are synthesized as inactive precursors that convert into mature enzymes within 2 min after wounding the plant when the latex is abruptly expelled. Papaya latex also contains other enzymes as minor constituents. Several of these enzymes namely a class-II and a class-III chitinase, an inhibitor of serine proteinases and a glutaminyl cyclotransferase have already been purified up to apparent homogeneity and characterized. The presence of a beta-1,3-glucanase and of a cystatin is also suspected but they have not yet been isolated (Azarkhan et al., 2003).
2.10.3 Purification of latex to obtain Papain

Literature regarding the purification of papain is on the rise. An array of methods from basic salting out to sophisticated chromatographic techniques, among which the aqueous two phase solvent extraction is quite popular, and ultrafiltration methods are tried to study this economically important enzyme (Zhiwen Bai et al., 2013; Senthikumar and Kumareshan 2012, Chaman Mehta et al., 2013, Baines and Broklehurst, 1979; Biswajit Paul et al., 2013; Nitsawang et al., 2006; Monti et al., 2000).

2.11 GLOBAL IMPORTANCE FOR ENZYMES

The increase in demand of consumer goods and increase in the number of applications of industrial enzymes, investments in the research and developments of industrial enzymes and the need of cost reduction and resource optimization in the production process are driving the global industrial enzymes market.

![Industrial Enzymes Market Size](image)

**Fig.8: Industrial Enzymes Market Size**

The key players in the market are BASF SE (Germany), E.I. du Pont de Nemours and Company (U.S.), Associated British Foods plc (U.K.), Koninklijke DSM N.V (The Netherlands), and Novozymes A/S (Germany) among others.
The industrial enzymes market is segmented on the basis of type into carbohydrases, proteases, non-starch polysaccharides and others which include phytase, lipases, catalase, and tannase. The carbohydrases segment is projected to grow at the highest Compound Annual Growth Rate (CAGR) of 7.5% from 2015 to 2020.

On the basis of application, the market is segmented into food and beverage, cleaning agents, animal feed, and others which include starch processing, textile & leather, biofuel. The market for food and beverage projected to reach a value of USD 2.0 Billion by 2020. [www.marketsandmarkets.com/food-and-beverages-market-research-6.html](http://www.marketsandmarkets.com/food-and-beverages-market-research-6.html) (18). The market has also been segmented on the basis of region into North America, Europe, Asia-Pacific, and the Rest of the World (RoW).

### 2.12 Industrial Enzymes Market

The industrial enzymes market was valued at USD 4.2 Billion in 2014 and is projected to grow at a CAGR of 7.0% from 2015 to 2020. In 2014, the market was dominated by North America. The Asia-Pacific region is projected to grow at the highest CAGR from 2015 to 2020. A list of all enzymes that are presently marketed in the EU includes 186 enzymes from 47 different catalytic types. The exploitation of new types of enzymes, improvements of enzyme properties and of the production process are the overall goals of innovation in the enzyme manufacturing industry.

### 2.13 MARKET FOR PAPAIN

The demand for papain is reflected by the following:

1. Mushrooming companies which are ready to provide licensed information on the market survey.

2. Networking services who are willing to set up papain producing factories.
3. The interest taken by various national and international governments encouraging growing of papaya.

4. The extent of research and concern shown for papaya cultivation (Biswas, 2010).

2.14 INDIAN ENZYME MARKET

The biotech industry in India accounts for about 2% of the global biotech markets. During the fiscal year 2010-11 the Indian biotech sector grew at 21.5% to reach Rs. 17,400 crores in revenues. India’s share in the world for industrial enzymes is about US $ 3387.30 million. The textile and leather enzyme segment are mature, while the detergent enzymes segment is in the growing stage. Almost 50% of the enzyme demand is in the pharmaceutical sector. Food and feed as well as leather and paper demand 5% each.

An overall view of the above facts claim the importance of enzymes and especially the plant enzyme. More significantly the great usage of the papain enzyme is broadly highlighted. This compilation suggests the justification of the work and it is the need of the hour.