1. INTRODUCTION

Enzymes are nature’s efficient entity. They are universal in occurrence. Life on Earth cannot be what it is without enzymes. Major metabolism of a living cell is facilitated with the aid of these biomolecules. They are the catalytic machinery of the living system.

The absence of enzymes leads to disastrous effects. Sustenance of life becomes a struggle for the cell. This same picture can be telescoped into multicellular organisms, all life forms and ultimately humans. They are the active molecules of utmost importance that play a critical role from the beginning to end and also after life. Without the enzymes decomposition cannot happen. Thus they maintain the life cycle on this planet, and are indispensable!

Techniques like fermentation, brewing, baking and tanning were part of early civilization even before man knew about microorganisms. Later, when microorganisms were discovered, their role in fermentation, brewing and baking and tanning was established beyond doubt. Intensive research on microorganisms gradually led to the discovery of little biomolecules that were responsible for all the essential day to day activities of man and they were named ENZYMES.

Mention of enzymes being used for cheese dates back to 400 BC in Homer’s Illiyad. It stated that milk turned into a semi solid substance when stored in the bag of the stomach of a calf lamb or kid immediately after slaughtering. In 1783, Lazzaro Spallanzani first mentioned the importance of this biomolecule in his work of biogenesis (spontaneous generation of microbes) where he mentioned that there is a life-generating force inherent to certain kinds of inorganic matter that caused living microbes to create themselves given sufficient time. Work on enzymes is continuously being done in various labs to understand their nature, structure and
function. In 1908, Otto Rohm introduced the application of pancreatic enzymes together with salts for bating in tanneries. Later in 1947, James Sumner proved the fact that enzymes are a type of protein that resulted after purification and crystallization of urease. It was in 1960 that Novo of Denmark started producing protease from \textit{Bacillus licheniformis} on a commercial scale. \url{http://dx.doi.org/10.1155/2013/329121} (1).

1.1 NATURE OF ENZYME

All known enzymes are proteins. They are high molecular weight compounds made up of chains of amino acids linked together by peptide bonds. They are globular in shape with a complex 3-D structure. Enzymes can be denatured and precipitated with salts, solvents and other reagents. They have molecular weights ranging from 10,000 to 2,000,000 KDa.

Many enzymes require the presence of other compounds, such as cofactors for their catalytic activity. This entire active complex is referred to as the holoenzyme. Apoenzyme (protein portion) plus the cofactor (coenzyme, prosthetic group or metal-ion activator) is called the holoenzyme.

1.2 FUNCTION OF ENZYME

Enzymes exhibit specific catalytic function and they are efficient in small amounts. They remain unchanged after the reaction and simply increase the speed of the reaction. Enzymes can increase the rate of reactions without increasing the temperature. They do this by lowering the activation energy. \url{https://books.google.com/books/.../Enzymes and their role in biotechnology.html} (2).

The substrate of an enzyme is the reactant that is activated by the enzyme. Enzymes are specific to their substrates. The specificity is determined by the active site. Unlike chemical catalysts most enzymes are specific. The active site of the
enzyme consists of 3-12 amino acid residues organized into a precise 3-D arrangement in a pocket in the protein. This site has a strong affinity for the substrate because of the chemical nature and structure. One part of an enzyme, the active site, is particularly important. The shape and the chemical environment inside the active site permits a chemical reaction to proceed more easily.

**The Lock and Key Hypothesis**

Fit between the substrate and the active site of the enzyme is exact like a key fits into a lock very precisely. The key is analogous to the enzyme and the substrate analogous to the lock.

A temporary structure called the enzyme-substrate complex is formed. Products have a different shape from the substrate. Once formed, they are released from the active site leaving it free to become attached to another substrate.

**1.3 ENZYME CLASSIFICATION**

The Enzyme Commission number (EC number) is a numerical classification scheme for enzymes, based on the chemical reactions they catalyze. Every enzyme code consists of the letters "EC" followed by four numbers separated by periods. Those numbers represent a progressively finer classification of the enzyme. Except for some of the originally studied enzymes such as pepsin, rennin, and trypsin, most enzyme names end in “ase”. [http://www.chem.qmul.ac.uk/iubmb](http://www.chem.qmul.ac.uk/iubmb) (3).

There are six main categories of enzymes, with their respective codes as fixed by the Enzyme Commission, which are as follows:

- **EC1 Oxireductases**: catalyze oxidation/reduction reactions.
- **EC2 Tranferases**: transfer a functional group from one compound to another.
- **EC3 Hydrolases**: catalyse the hydrolysis of various bonds.
• EC4  Lyases : cleave various bonds by means other than hydrolysis and oxidation

• EC5  Isomerases : catalyze isomerization changes within a single molecule

• EC6  Ligases : join two molecules with covalent bonds

1.4 PREFERENCE OF ENZYMES OVER CHEMICALS

Though enzymes were in use as early as 3000 years ago, why are they given such great importance today? The answer lies in the following facts:

1. Enzymes accelerate the reactions a million fold thus saving time and energy immensely.

2. They operate under mild temperature and pressure, in contrast to the usual high end parameters which chemicals require.

3. They are a safer alternate to hazardous chemicals used by the major industries of the world such as textile, leather, detergent, food, etc. (Kiro Mojsov, 2013).

4. As enzyme - substrate reactions can be clearly identified various physical parameters can be controlled and regulated easily.

5. A very prominent feature they carry is that they are less or non-polluting and easily biodegradable.

6. Commercial enzyme production has rocketed in the last century in response to the expanding markets and ever increasing demand for newer biocatalysts.

7. The list of industries using enzymes is mind boggling. From tanneries to gene therapy enzymes are applied and life is made easy by utilizing this
nature’s bounty. Following are some of the major industries that benefit by the enzymes: bakery, brewery, tannery, food, fish and poultry feed, dairy, edible oil extraction, fruit juice extraction, cosmetic, pharmaceuticals, organic chemical synthesis, detergent, personal care, paper and textile industry, biofuel industry, oil and gas drilling, biopolymers, genetic engineering and much more. https://www.novozymes.com/en (4).

1.5 SOURCES OF ENZYMES

Biologically active enzymes can be extracted from any living organism. For example, protease can be extracted from a microorganism, Bacillus (serene protease), or from cow pancreas (pancreatin) or from a fruit, pine-apple (bromelain).

Currently there are three major sources of enzymes, namely, animal, plant and microbes. Over half of the enzymes used in the industries are from fungi and over a third are from bacteria and the remaining is from animal ( 8% ) and plant ( 4% ).

Though the percentage of plant enzyme is comparatively less, till today it is used world over, in food, beverage, poultry and fish feed, pharmaceutical, cosmetics, tanneries and so on. A few industries sustain the production of enzymes to meet the continual demand of the world market.

The most popular plants from which enzymes are derived are: Kiwi fruit, Barley, Pineapple, Fig, Soybean, and Papaya. Greenberg, (1955) quotes plant enzymes from the following sources (Table 1).

1.6 GLOBAL MARKET FOR ENZYMES

There are about 3000 known enzymes in the world and about 150-170 enzymes have industrial use. The continuing demand for enzymes in this modern era has influenced the global economy significantly. The value of industrial enzymes has
<table>
<thead>
<tr>
<th>Enzyme name</th>
<th>Plant species</th>
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<tbody>
<tr>
<td>Papain</td>
<td><em>Carica papaya</em></td>
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<tr>
<td>Chymopapain</td>
<td><em>Carica papaya</em></td>
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<td>Ficin</td>
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<td>Mexicanin</td>
<td><em>Pileus mexicanus</em></td>
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<td>Asclepain</td>
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<td>Bromelain</td>
<td><em>Ananas sativa</em></td>
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<td>Pinguinain</td>
<td><em>Bromelia pinguin</em></td>
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<td>Taberna montanain</td>
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<td>Soyin</td>
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<td>Solanin</td>
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<td>Euphorbain</td>
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<td>Hurain</td>
<td><em>Hura crepitans</em></td>
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<td>Pomiferin</td>
<td><em>Maclura pomifera</em></td>
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<td>Arachain</td>
<td><em>Arachis hypogea</em></td>
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Fig. 1: Industrial Enzymes
been calculated at about $3.3 billion in 2010 and is expected to rise at a pace of 3-5 per cent annually. The leather market had the maximum sales followed by bioethanol market. Food, animal feed, detergents, paper and textile are projected as the prospective industries of the future (Fig.1).

About 15 companies dominate the world in enzyme production and Novozymes ranks first by holding a 45% share of the market. https://www.eolss.net/EOLSS (5).

India too is not short of its potentials in the enzyme industry but still is in its infancy. Though its share is very less, it has its presence in the international market which is estimated to be about US $3390 million (Binod et al., 2013). There is a demand for enzymes in the sub-continent too for detergent, textile, pharmaceutical, food and feed, paper and leather industries. There are a couple of Biotechnology companies manufacturing pharmaceutical enzymes, and a few industrial enzyme companies, which produce papain.

There was a boom in the papain industry in the 1990’s and then a slump and again it has gained momentum. To fillip the Indian industries the present study is projected in the area of manufacturing the papain enzyme. The downstream processing of the enzyme is taken into consideration and ways to cut cost and simplify the traditional protocols is researched. In short, this research is aimed at bridging the gap between sophisticated science and layman's rough techniques and thus creating a moderate method to benefit the producers and consumers.

1.7 PAPAYA AND PAPAIN ENZYME

The papaya (Carica papaya L.) is native to Central America and is grown in many continents. Its fruits are consumed either raw or ripened and is now cultivated as a fruit crop in most tropical countries and many subtropical regions of the world. South American countries such as Mexico, Brazil, Peru, Venezuela, Asian countries
like India, Sri Lanka, Taiwan, Thailand, Philliphines and African nations like Uganda, Kenya, Tanzania, Ghana and Zaire cultivate papaya.

The plant belongs to the family Caricaceae. Its classification is as follows: Division: Magnoliophyta, Class: Magnoliopsida, Subclass: Dilleniidae, Order: Violales.

The genus name Carica is derived from the latin name for a kind of fig, which the leaves and fruits of Carica papaya resemble. The specific epithet papaya comes from the common name for the fruit (Du Puy and Telford, 1993).

Carica papaya L. is a fast growing arborescent herb with a short life. The plant is short-lived, fast growing, woody, herb-like tufted tree that can grow up to 10 meters in height. https://www.oecd.org/about/34711139.pdf (6). It generally branches only when injured. It is a dicotyledonous, polygamous (having male, female or hermaphrodite flowers on the same plant), diploid species with small genome. Carica papaya is the only species within the genus Carica.

Papaya has several industrial uses. Biochemically, its leaves and fruit are complex, producing several proteins and alkaloids with important pharmaceutical and industrial applications (El Moussaoui et al., 2001). The papain enzyme gradually gained attention for its protease activity from the time of its discovery. Today it is routinely applied in the industries through out the world. Though chymopapain is present in a higher percentage, industrially papain has a greater demand. Latex is present in the leaves, shoot and fruits. For commercial purpose the latex of raw fruits is preferred because it is rich in enzymes.

1.7.1 Cultivation of Papaya for papain

Special varieties of Papaya are hybridized for papain production. Tamilnadu Agricultural University has developed CO5, CO2 and CO6 varieties especially for latex production. http://agritech.tnau.ac.in/horticulture/horti_fruits_papaya.html (7). The yield of crude papain is as follows: CO2 - 600 kg/ha - CO5 - 800 Kg/ha. Some
hybrids from Taiwan too prove successful for latex extraction. Red Lady, a Taiwanese variety-the latex yield is around 1.5 tonnes an acre.

The seedlings should be prepared in raised beds in sunny areas. Female plants should be more in number to get high yield. The soil should be rich loamy, deep and well drained. The papaya is very sensitive to even short periods of flooding. Irrigation should be provided during dry spells because a fluctuating water supply may cause growth retardation, flower abortion and dropping of young fruits. FAO, 2003 http://www.itver.edu.mx (8).

In 2 ½ years on one acre of papaya plantation gives 1700 kg of papain from its latex, 100 tonnes of unripe fruit or 50 tonnes of ripe fruit. The Hindu -money minting papaya agritech.tnau.ac.in/ success_stories/ pdf/2010 (9).

1.7.2 Latex Collection

The collection of latex is a very arduous task demanding meticulous labor. The latex should be tapped from immature papaya fruits which are 75 to 90 days old. On the selected fruit, incisions (cut) are given with a razor blade or stainless steel knife. The cuts should be given from stalk to tip of the fruit. The depth of the cut should not be more than 0.3 cm. Four such cuts are given, spaced equally on the fruit surface. The latex is tapped early in the morning and completed before 10.00 a.m. Tapping is repeated four times on the same fruit at an interval of three days. The cut should be given on the fruit surface in places not covered by previous cuts. It is collected in resin sheets or aluminum trays. It is scooped and packed in plastic bags and sent to cold storage. The milk becomes like wax in a short time on standing, within 4 to 6 hours. The latex shows no apparent putrefaction for a period of about 4 hours but subsequently it deteriorates and becomes alkaline. http://agritech.tnau.ac.in/ horticulture/horti_index.html (10).
1.7.3 Latex Composition

The milky latex is slightly acidic fluid composed of 80% of water. It contains sugars starch grains minerals (S, Mg, Ca, K, P, Fe, Zn), alkaloids, isoprenoids, lipidic substances and proteins including enzymes like lipases, cellulases, and cysteine proteases (papain, chymopapain). The latex is important in defense against insect herbivores and in tissue and organ formation and pith differentiation (Konno et al., 2004).

1.8 PAPAIN ENZYME

Papain (EC3:4:22:2) enzyme is one of the most widely used enzymes in various industries. From the time of its isolation by Balls and Thompson (1937) research on this enzyme has branched out on various aspects from farm to lab. High latex yielding variety has been reported from the state of Tamilnadu, India. Studies to combat the foliar diseases, boron deficiency and other diseases to improve production have been suggested by Nelson (2016). http://hawaiiplantdisease.net/Publications.php (11). Genetically modified plants to withstand PRSV (papaya ring spot virus) were extensively studied by Ferreira et al., (2002). Breeding studies using Irap Markers were done by Rashid et al., (2014) and cloning of this enzyme was done by Cohen et al., (1986). Many researchers performed recombinant studies on the enzyme and one prominent study is that of Taylor et al., (1995).

1.8.1 Characteristics

Papain (EC 3:4:22:2) is also known as papaya peptidaseI. It has a molecular weight of 23,000 daltons, an optimum pH 6.0 - 7.0 and temperature in the range of 65°C and 80°C. The isoelectric point is 9.6 and is stable in its crystal form at 5°C. Its stabilizing agents are EDTA, cysteine and dimercaptoetanol (Chu Chi Ming et al., 2002).
1.8.2 Activity

Papain has a wide range of activity. It is a cysteine protease. It is characterized by its ability to hydrolyze large proteins into smaller peptides and amino acids. It degrades most protein substrates more extensively than pancreatic proteases. It is also an esterase. Papain breaks down the intracellular matrix of cartilage and it is activated by cysteine, sulphide and sulphite. Activity is enhanced in the presence of heavy metal chelating agents such as ethylene diamine tetra acetic acid (EDTA). General reaction conditions are pH 6-7 at temperature of 20°C to 37°C.

1.8.3 Uses of Papain

The uses of papain runs into a lengthy list. The history of its use indicates it was a folk remedy and still exists among some tribes of the world. Modern era has exploited its benefits to the maximum. There is not an industry that goes without using papain. The wide application has raised it to a global status and is referred to as a money minting enzyme.

Medicine

Papain is also an ingredient in a dental product called Papacarie used to treat dental caries without cutting and drilling. Papain in this product breaks down the damaged tissue inside the cavity without damaging the normal tooth structure. This reduces or eliminates the need for drilling and is less damaging to teeth. It is also less painful to the patient. In addition, papain is an ingredient in enzymatic cleaners used to clean contact lenses.

Cosmetics and Personal care

Papain is also an ingredient in some skin care products. Papain can have some good influence on collagen, by which it can assist in removing freckles and enable the skin to appear even and white. It protects the cutin, accelerates metabolism of
epidermal cells and makes the skin tender. Some practitioners believe that papain helps to open up clogged pores and is effective for addressing blemish prone skin. This enzyme from the papaya fruit also has anti-inflammatory and anti-bacterial properties. Because of its ability to remove dead skin cells, papain is found in a variety of exfoliating skin care products including facial scrubs, body cleansers, facial masks and peels. Papain is added in toothpaste, mouthwash and dentifrice. It can clean mouth and remove tartar and dental calculus.

**Food Industry (General)**

Papain can hydrolyze the macromolecular protein of food into small peptides and amino acids by its enzymatic reaction. It can be applied to hydrolyze animal protein and plant protein. Papain can be used for meat tenderizing, brewing, clarification, preparation of beverages, biscuits. Papain can improve the nutritional value of health food and help the digestive system assimilate food easily.

**Papain in Dairy**

The processed enzyme is used in cheese making (Mahajan and Chaudary 2014). Papain is also used as a hydrolysate in dairy to process whey proteins which are used in the diet as non-pharmacological approach to prevent and treat arterial hypertension (Silvestre et al., 2012).

**Biscuit Industry**

Papain can hydrolyze the macromolecular protein of the biscuit making ingredients into peptides and amino acids thereby reducing the dough’s gluten. As a result, the plasticity and physicochemical property as well as the color of the biscuit is improved. Use of papain can reduce 10%-25% sodium metabisulfite’s dosage thus the residual quantity of SO$_2$ is reduced.
**Papain in Cattle Fish and Poultry feed**

Papain can be put in the forage’s formulation or directly put in the forage. It is helpful for promoting digestion in animals, improving the utilization of the feed, cutting the cost of feeding and accelerating the growth and increasing the nutritive value.

**Pharmaceutical Industry**

Tablets and capsules made from papain can help ease swelling, alleviate inflammation, improve immunity, promote digestion. Papain can be used for blood group identification. It can treat gynecological diseases, glaucoma and insect bites.

**Textile Industry**

Papain is used for wool shrink proof. Wool treated with papain will be more tensile and softer. Papain can be applied on degumming and refining of silk too.

**Leather Industry**

The various important processing methods involved in the leather manufacture are curing, soaking, liming, dehairing, bating, degreasing and tanning. All these successive steps in the leather production are facilitated by the use of enzymes. Depilatory agents made with papain will make the leather more shiny. Papain together with ultra sound gives better results for bating (Sivakumar et al., 2011).

**Papain in Bioengineering, Bioprocessing and Genetic engineering**

**Papain used to obtain peptide derivatives**

Papain-catalyzed esterification of fibroin peptides was investigated at the individual peptide level using liquid chromatography-mass spectrometry with selected ion monitoring. This hydrolysis is useful to create novel peptide derivatives, like a peptide detergent or a self assembled nano peptide structure. Also the substrate
specificity of papain and the unique repetitive sequence of fibroin generated the hydrolysate composed of even number of amino acids at a high percentage using MALDI-TOF and ESI mass spectrometric analysis. This study provides a clue to understand the fate of peptides in a protein hydrolysate (Jeong and Hur, 2010).

**Papain in immunology**

Antibody purification too is enhanced with the utilization of papain (Galaev and Mattiasson, 2001).

**Papain in synthesis of biomolecules**

Papain is used to catalyze the synthesis of amino acid derivatives to produce biosurfactants that are used as antimicrobial agents and to treat lung diseases (Dunford, 2012).

**Papain in drug design**

Most of the amino acid residues that are involved in the binding of papain are conserved in cathepsin L. This publicly available high resolution structure has provided an excellent model for the successful design of highly active and specific cathepsin L inhibitors (Ezekiel and Mamboya, 2012).

**Papain and molecular modeling**

A structure-based rational design approach has been used to improve the thermostability of papain without perturbing its enzymatic activity by introducing three mutations at its interdomain region. (Choudary et al., 2010)

**Papain gene as a reference gene for Genetically Modified Papaya**

Genetically modified (GM) papaya lines have been approved for commercialization and are widely cultivated in many countries. In Korea a duplex PCR analysis was developed to detect a genetically modified (GM) papaya (Kim et al., 2010).
The above is only an outline of the uses of this hyper potential enzyme and the current research justifies the need to find improved methods to enhance the production of this wonder enzyme for the beneficial needs of the 21st century man.