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Development of eco friendly liquid laundry detergent with fruit peels and protease from *Bacillus subtilis*

By

Deepthi Sri. R

21PBC004

II M.Sc BIOCHEMISTRY

Department of Biochemistry, Biotechnology and Bioinformatics

A thesis Submitted to Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore-641 043.

In partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE IN BIOCHEMISTRY

MAY 2023

Certificate

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DEEPTHI SRI R

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Introduction

1.0 INTRODUCTION

Enzymes are macromolecular organic catalysts. The chemical processes are accelerated by enzymes. The molecules that act upon enzymes are known as substrates. A product is produced due to the interaction between the enzyme and substrate. Enzymes are essential for almost all cellular processes in living things. Enzymes are required for each metabolic pathway step to catalyse the reaction. Enzymology is the field of research dedicated to enzymes.

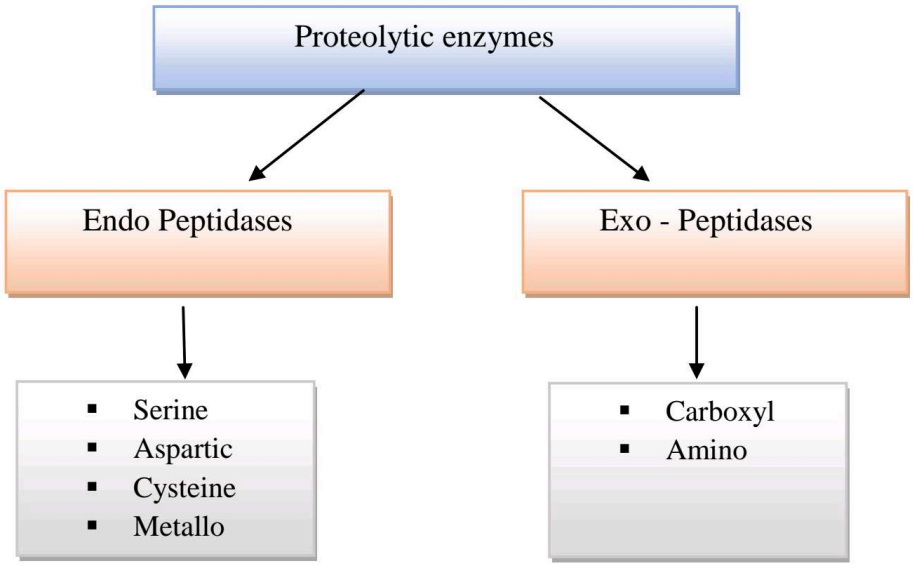
All enzymes are proteins, although some exceptions exist, such as catalytic RNA molecules called ribozymes. Because of their distinct three-dimensional structures, enzymes have a distinctive character. When enzymes speed up a reaction, the activation energy is reduced. In terms of chemistry, neither enzymes consume substances in chemical processes nor do they change the equilibrium of the reaction. Different Molecules can affect the way an enzyme works. Inhibitors are substances that lower chemical activity, whereas activators enhance movement (Cavaco-Paulo and Gubitz, 2003 and Purich, 2010).

For instance, a few enzymes are used industrially to manufacture of antibiotics. Enzymes are used in some household items to speed up chemical reactions. Enzymes in organic washing powders separate proteins, remove starch or fat stains from clothing, and break down proteins into smaller molecules in meat tenderizers to make the meat easier to chew or bite (Hasan *et al*., 2010 and Bekhit *et al*., 2014).

The class of enzymes known as proteases is primarly helpful in industrial applications. All organisms naturally contain protease enzymes, which account for 1–5% of the total protein composition (Singh *et al*., 2012). The protease industry is the third largest segment of the enzyme market, accounting for 60% of global sales (Akcan and Uyar, 2011).

There are several types of proteases, including serine, cysteine, metallo, asparagine, glutamate, and threonine, as shown in the Figure 1. Proteins can be broken down into smaller peptides or amino acids by proteases, which are enzymes. Proteases are also known as peptidases, proteinases, or proteolytic enzymes because they can hydrolyze the peptide bonds in proteins (Sharma *et al*., 2015). The regulation of protease is aided by nutritional signals in bacteria and fungi, and it plays a significant part in the carbon and nitrogen cycles in the recycling of proteins (Sims *et al*., 2006).

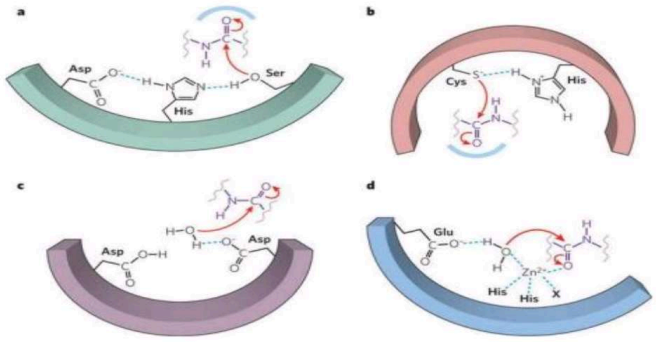
FIGURE 1: CLASSIFICATION OF MICROBIAL PROTEASE BY THEIR PROTEOLYTIC ACTIVITY AND ACTIVE SITE



Source: Gemechu *et al*., 2020

Both enzymatic and chemical methods can be used to cut polypeptides. Figure 2 shows the active site of proteases which hydrolyze peptide bonds; this process is known as proteolysis, and the by-products are free amino acids and peptide fragments (Erez, 2009). Proteases are one of the ingredients found in all types of detergents, including those used in households and cleaning solutions for denatures (Sangeetha *et al*., 2011).

FIGURE 2: ACTIVE SITE OF PROTEASES



(a) Soluble serine proteases; (b) Cysteine proteases ; (c) Aspartyl proteases; (d) Metalloproteases.

Source: Archana *et al*., 2018

Plants, animals, and microscopic creatures all have proteases (Kumar *et al*., 2012). Additionally, plant proteases are involved in the control of photosynthesis (Zelisko and Jackowski, 2004). Microorganisms are preferred for industrial production so that large-scale production of enzymes can be achieved. Depending upon the type of strain, pH, temperature, time of incubation, method of cultivation, the composition of medium, cell growth, nutritional requirements, metal ions and thermo stability, the production of microbial protease differs (Kumar *et al*., 2012).

The majority of enzymes derived from microorganisms are *Bacillus species*, but only a few numbers are suggested for use in commercial production. Hay *bacillus* and Grass *bacillus* is another name for *B.subtilis* is primarily found in soil. They are organisms with rod form. They can endure harsh environmental conditions because they can generate a hard, protective endospore. Both free-living and pathogenic species of *Bacillus* are obligate aerobes or facultative anaerobes (Dubal *et al*., 2008 and Sekhon, 2010). Numerous industrial enzymes and specialized compounds are produced using *Bacillus subtilis* (Esakkiraj *et al*., 2009; Chu, 2007 and Gupta *et al*., 2002).

Bacillus subtilis was chosen as the biological agent in the submerged fermentation because of its ability to produce proteases and because it displayed the highest level of proteolytic activity. Waste is transformed into useful biomass by microbes, and their enzymes are a recent development. The demand for this enzyme synthesis is constantly increasing, requiring the development of new protease-producing microbes and advanced fermentation techniques (Rathakrishnan *et al*., 2012). Numerous scientific studies have revealed that the alkaline protease activity was at its highest under ideal circumstances. Additionally, the protease was able to hydrolyze the gelatin in X-ray film and remove blood stains from cotton fabrics. The proteases therefore, indicated possible use in the detergent and photography industries (Jafari *et al*., 2023).

Alkaline proteases account for 60–65% of all industrial markets worldwide (Zanphorlin *et al*., 2010 and Sawi *et al*., 2008). Alkaline proteases are used in many different industrial products, including detergents, food, medicines, and leather. Additionally, a number of microbial strains were tested for alkaline protease synthesis utilising skimmed milk agar medium, agar media, and the gelatin hydrolysis method. Optimum media, pH, ideal incubation, and temperature are only some of the variables that must be considered in order to

maximise the production of enzyme from the source. The protease enzyme is used to create pharmacological medicines that fight fatal diseases including AIDS and cancer (Khusro *et al*., 2018).

The main source of important vitamins and minerals is fruit. Fruits are a significant source of antioxidants and can thus be used as a natural medicine for a wide range of illnesses, including the healing of wounds, the relief of itchy skin, and the protection of our skin from sun damage. Large-scale industrial processing of fruits and vegetables results in the production of wastes and by-products that are harmful to the environment. Consequently, they must be used or managed (Duda-Chodak and Tarka, 2007). In addition to being consumed, fruits are utilised in toothpaste, shampoo, and cosmetics.

Lemon is a member of the Rutaceae family and is regarded as a significant medicinal plant. They are primarily grown for their alkaloids, which have antibacterial and anticancer qualities. Lemon's leaves, stem, root, and blossom all exhibit clinically significant antibacterial activity in crude extracts, according to reports (Kawaii *et al*., 2000). Lemon trees can reach heights of ten to twenty feet. The leaves of these are small, prickly trees are placed alternately along the stem. The lemon leaves appear to be a dark green tint. Lemon blossoms have five petals and are white in colour. This flower is derived from the "Pink Lemonade" variety of lemon. This cultivar's fruit is striped and its leaves are variegated (Mohanapriya *et al*., 2013).

The flavonoids in *Citrus* offer a wide range of benefits, including antiviral, anticancer, antifungal, and antibacterial actions (Burt, 2004 and Ortuno *et al*., 2006). For two reasons, limonene is utilized in cleaning products: it has a nice scent and works as a solvent to dissolve dirt (Etta-francis *et al*., 2022).

The fruit known as papaya, or *Carica papaya* Linn, is a member of the Caricaceae family and is often called paw-paw. Papayas have gained worldwide acclaim for their culinary and nutritional benefits. The qualities of papaya are well-known in traditional medicine, and other parts of the plant are frequently employed as medicines. Papaya has been acknowledged for its biological activity and medicinal uses throughout the past few decades, and it is currently regarded as an important nutraceutical fruit plant.

Due to the abundance of therapeutic qualities found in papaya, it is used in the treatment of numerous ailments. The *Carica papaya* plant's various parts, including the leaves, seeds, latex, and fruit, are believed to have medicinal potential. The papaya's stem, leaf, and fruit are all rich in latex, and the unripe fruit's latex is notably rich in enzymes like papain and chymopapain (Yogiraj *et al.,* 2014). Papain, an enzyme that also serves as a protease in papaya peels, acts as a stain-removing agent (Srujana *et al*., 2020 and García-Villegas *et al*., 2022).

The first conclusive evidence of soap-like substances was discovered around 2800 BC. The earliest people to create soap were the ancient Greeks and Romans, as well as the Babylonians, Mesopotamians, and Egyptians. By combining fat, salts, and oils, they created soap. Soaps were used for cleaning kitchen utensils, bathing, personal hygiene, and even for medical purposes. Currently, soaps come in a wide variety and are created for a variety of uses, including industrial, commercial, and personal use.

Both handcrafted and commercially produced soaps are used to wash clothes, dishes, and automobiles (Fonseca *et al*., 2019 and James *et al*., 2000). Clothes, designs, fashion, human preferences, and expectations are all changing swiftly as time goes on. To accommodate consumer demand, new technologies and production techniques are being developed for the clothing industry. Technology advancements in the laundry room have made it possible to satisfy contemporary needs, to have a silky texture and to overcome a fading look in knit cloth. The use of enzymes is quite efficient. Additionally, enzyme are utilised as a biocatalyst and a bio polish. Numerous studies examine how enzyme washing modifies knit fabrics made entirely of cotton (Jahan *et al*., 2022).

The saponin-rich soap nut, is also referred to as reetha, soapberries, or cleaning nuts. One of the main components in hair treatment, particularly in Ayurveda, is soap nuts. They produce soft suds that form lather when they come into contact with water. It's interesting to note that soap nut advantages extend beyond hair. There are numerous uses for the seeds. For example, they can be used to polish the jewellery and sanitise it. They also provide some advantages for the skin and general well-being. The fact that they are natural, affordable, reusable, and eco-friendly is their finest feature (Anjali and Divya, 2018).

Ocimum basilicum L. is the official botanical name for sweet basil. It belongs to the Lamiaceae family. Historically, essential oils from *Ocimum basilicum*'s leaves and flowers have been extensively utilised in food processing systems as flavouring agents and in the pharmaceutical industry for therapeutic purposes (Telci *et al*., 2006).

Essential oil is an evaporating, volatile material that is insoluble in water. Essential oils, which are frequently used in fragrances, medications, and cosmetics, can be extracted from any part of the plant, including the leaf, branch, root, stem, flower, and seed. Distillation or extraction techniques can be used to isolate them from plant tissue. However, distillation is favoured by several companies as a process (Mindaryani and Rahayu, 2007).

The present study entitled "**Development of eco friendly liquid laundry detergent with fruit peels and protease from *Bacillus subtilis*"** is carried out with following objectives:

- To isolate, partially purify and characterize the protease enzyme from *Bacillus subtilis* and the production of liquid laundry detergent.
- To extract essential oil from *Ocimum basilicum* leaves.
- To prepare extract from lemon peels, papaya peels and soap nuts.
- To analyse the product with various stains.

Review of literature

2.0 REVIEW OF LITERATURE

Socioeconomic factors are altering the global environment. These elements include the waste management, dairy processing, leather, and food processing industries (Godswill and Somtochukwu, 2017). Many industrial operations harm the environment, making them unpopular with society. Numerous societies reject the leather industry because of its air pollution and offensive odour (Hadush *et al*., 2017). The leather industries require chemicals at various stages of their production, and different raw materials are used. The best way to remove odour and air pollution is to use an enzyme rather than a chemical (Amare *et al.,* 2019).

Enzymes play a significant part in many applications, the things we utilise for fuel, food, housing, medication, cleaning supplies, dairy products, etc. An enzyme's key benefits include its low energy consumption and ability to lessen environmental pollutants (Tan, 2016).

This review of the present work entitled "**Development of eco friendly liquid laundry detergent with fruit peels and protease from *Bacillus subtilis*"** focus on the following topics

2.1 Proteases

2.2 Protease Sources

2.2.1 Plant Sources

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2.1 PROTEASE

Protease can be produced by all living things, unlike other enzymes that can only be produced by few organisms. Proteolytic enzymes can come from plants, animals, or microbes. At some point, the complete material has been cited as the source. The finest sources of proteolytic enzymes are plants and animals, although it is challenging to use these sources as long-term enzyme supplies (Singh *et al*., 2004). It is not advisable to employ plants and animals as a source on a big scale because they are naturally limited in number. One cow's

death for one rennin or chopping of a huge plant for papain may harm the environment and ecology.

On the other hand, we can grow microorganisms in vast quantities on a small surface and in minimal media. The best source of enzymes became the microorganisms as a result. Again, microbes are more effective than plants and animals at producing enzymes. These elements suggest that the best providers of enzymes on this expanding planet are microorganisms (Rao *et al*., 1998).

Proteases are a group of enzymes that catalyse the hydrolysis of peptide bonds in proteins. They are also referred to as proteinases or proteolytic enzymes. Proteases comprise a sizable set of enzymes that fall within the hydrolase category. They are found everywhere in nature and play a big part in both physiological and commercial uses. Nearly all plants, animals, and microbes contain these enzymes (Rani *et al*., 2012).

The majority of protease enzymes generated by microorganisms are secreted into the fermentation broth in an extracellular form. Microbial protease can be chosen over plant and animal protease thanks to these downstream pathways that enable bacteria to manufacture pure enzymes in huge quantities (Gupta *et al*., 2002 and Gaur *et al*., 2014). The majority of microorganisms that produce proteolytic enzymes are bacteria and fungi (Raval *et al*., 2014).

High catalytic activity and substrate selectivity are characteristics of proteases. About 2% of the human genome and 1–5% of the genomes of pathogenic organisms are made up of proteases. Indeed, based on an extrapolation by Rawlings and his colleagues, the number of proteases may be at least twice as high, controlling the activation, synthesis, and turnover of proteins to regulate the majority of physiological processes. Proteases are essential regulators of all life processes, including conception, birth, digestion, development, maturation, ageing, and even death (Sharma *et al*., 2017).

Proteases is a broad term coded as EC 3.4.X.X. These enzymes come in two different categories, according to Grassman and Dyckerhoff. One kind, categorised as a proteinase or endopeptidase, has an endo mode of action and functions best on intact proteins. Consequently, these enzymes often within the molecule cleave peptide bonds of non-terminal amino acids. Exopeptidases of the second class, such as carboxypeptidases, are called amino

peptidases, are enzymes that dissociate peptide bonds between amino acids at the N or C termini of polypeptide chains (Contensini *et al*., 2018).

On the basis of catalytic process and the presence of amino acid residues at the active site, proteases can be divided into aspartic, cysteine, glutamic, metallo, asparagine, serine, threonine, and proteases with mixed or unknown chemical action mechanisms (Motyan *et al*., 2013).

Proteases are divided into three classes based on their acid-base nature: neutral, acidic, and alkaline proteases. Since they are the main source of acid proteases, fungi function best in the pH range of 2.0 to 5.0. As alkaline proteases function best at pH levels of 8 and above, microorganisms are the leading suppliers of these enzymes. Several industries depend on the proteases that microorganisms produce (Alnahdi, 2012).

For the proliferation of viruses, bacteria, and parasites, as well as the development of infectious diseases, proteases are also crucial. They are also necessary for transmitting disease in all insects, animals, and other organisms, as well as in human and animal hosts for the mediation of diseases. Proteases are essential targets for medical research and medication development because of their crucial regulatory roles throughout the life cycle. Currently, known hereditary/genetic disorders occur from single amino acid mutations in more than 50 human proteases (Sharma *et al*., 2017).

Protein turnover, cell division, the blood clotting cascade, signal transmission, the processing of polypeptide hormones, apoptosis, and the life cycles of many pathogens, including the replication of retroviruses, are all regulated by proteolytic enzymes.

Proteases are also used in a variety of research applications, including peptide synthesis, digestion of unwanted proteins during nucleic acid purification, synthesis of Klenow fragments, tissue dissociation in cell culture experiments, the preparation of recombinant antibody fragments for research, diagnosis, and therapy, and structural studies investigating the relationships between structure and function (Motyan *et al*., 2013).

The bulk of commercial enzymes in use today degrade various natural compounds through a process known as hydrolysis. Due to their widespread use in the dairy and detergent industries, proteases continue to be the most common type of enzyme. Numerous

carbohydrases, mainly amylases and cellulases used in the starch, textile, detergent, and baking industries, make up the single largest group. In addition to the food, paper, pharmaceutical, leather, and textile industries, microbial enzymes have also made their way into other industries and are quickly displacing traditional methods due to the advantages of high-quality products, greater efficiency than traditional methods, and reduced environmental harm (Gurung *et al*., 2013).

2.2 PROTEASE SOURCES

Proteases are found in all species, including viruses, eukaryotes, and prokaryotes. Many physiological responses, ranging from the straightforward digestion of dietary proteins to tightly controlled cascades, are affected by these catalysts (e.g., the blood-thickening cascade, the supplement framework, apoptosis pathways, and the invertebrate prophenoloxidase - initiating cascade).

Fortunately, proteins may be extracted from living cells and used for catalysis without affecting their physiological state. Commercial proteases are produced by fermenting animal tissues, plant cells, and microbial cells. Proteases are found across the entire biological source. The following is a list of some of the sources of proteases (Patel, 2017 and Zhang *et al*., 2013).

2.2.1 PLANT SOURCES

Vegetarian rennet, which contains plant-based protease, has been used to make kosher and halal cheeses in Europe and the Middle East for many years. Vegetarian rennet from *Withania coagulans* has been used for a long time as an Ayurvedic treatment for digestion and diabetes in the Indian subcontinent. For instance, it is used to produce paneer. Many proteases, a significant portion of which are unknown are encoded in plant genomes. Those with distinguishing characteristics are primarily concerned with controlling development (Van-der-Hoorn, 2008). Moreover, plant proteases are involved in the control of photosynthesis (Zelisko *et al*., 2004).

Many factors, including the availability of land for cultivation and the suitability of climate conditions for growth, influence the utilisation of plants as a source of proteases (Rani *et al*., 2012). Whereas bromelain is obtained from the stem and juice of the pineapple, papain is found in the latex of *Carica papaya* fruits. But they are laborious, time-consuming tasks (Kumar *et al*., 2016).

2.2.2 ANIMAL SOURCES

Proteases are utilized by an organism throughout its life for various metabolic functions. The digestion of dietary proteins is facilitated by serine found in the duodenum (trypsin and chymotrypsin) and acid proteases made in the stomach (such as pepsin). Blood serum contains proteases (thrombin, plasmin, Hageman factor, etc.) essential for blood clotting, clot dissolution, and immune system health. Elastase and cathepsin G are two additional proteases that are present in leukocytes and have a variety of roles in metabolic activity. Like pit viper haemotoxin, which interacts with the victim's blood clotting cascade, several snake venoms are also proteases. Proteases examine other proteins that play crucial roles, including hormones, antibodies, or additional enzymes. It is one of the fastest regulatory processes for "switching on" and "switching off" in an organism's physiology (Clausen *et al*., 2011).

The most well-known proteases of animal origin include rennin, pepsin, chymotrypsin, and pancreatic trypsin. Trypsin is the primary intestinal digestive enzyme in the hydrolysis of dietary proteins. Chymotrypsin is made from animal pancreatic extract. In its purest form, chymotrypsin is a costly enzyme exclusively used in diagnostic and analytical procedures. Every vertebrate's stomach contains pepsin, an acidic protease. All breastfeeding mammals produce rennet from the stomach, a pepsin-like protease, as an inactive precursor. By using the enzyme, it is changed into active rennin. It has been widely employed in the dairy industry to produce stable curd with good flavour (Jisha *et al*., 2013).

2.2.3 MICROBIAL SOURCE

The inability of plants and mammals to meet the world's demand for proteases has increased interest in microbial proteases. Due to their wide metabolic variety and genetic susceptibility, microorganisms are an excellent source of enzymes. Around 40% of all enzyme sales globally are accounted for microbial proteases. Proteases from microbes have

nearly all the desired properties for their biotechnological applications, they are preferable to enzymes from plant and animal sources (Ali *et al*., 2017).

The protease enzyme produced by bacteria hydrolyzes peptide bonds, breaking proteins into amino acids. Protease regulation, produced by bacteria and fungi, is aided by nutritional signals in these species. Protease plays a significant part in the carbon and nitrogen cycles in the recycling of proteins (Sims *et al*., 2006). The effect of nutritional signals of protease activity in many species found in soil is noticed at the microbial community level, as the proteins are broken down due to carbon, nitrogen or sulphur imitational responses (Sim and Wander, 2002). Protease, a bacterial enzyme that breaks down unfolded or misfolded proteins, can influence the overall quality of proteins.

2.2.3.1 FUNGI

The main sources of fungal proteases include a number of *Aspergillus species*, including *A.candidus*, *A.flavus*, and *A.fumigatus*, among others. Moreover, it can alter food proteins (Kumar *et al*., 2016).

2.2.3.2 BACTERIA

Because of their excellent production capacity and catalytic activity, bacterial proteases are essential commercially in the laundry, food, leather, and silk industries. The primary sources of bacterial proteases are *Bacillus species*. Moreover, key sources of bacterial proteases include *Alteromonas sp*., *Brevibacterium linens*, *Lactobacterium helveticus*, and others (Kumar *et al*., 2016).

2.2.3.3 VIRUSES

Viral proteases have become more significant due to their role in the processing of viral proteins resulting in deadly diseases like cancer and AIDS. Many viruses contain serine, aspartic, and cysteine peptidases (Kumar *et al*., 2016).

2.3 ALKALINE PROTEASE

Alkaline proteases are enzymes active at alkaline pH levels with optimal pH ranges between 9 and 11. In the manufacture of industrial alkaline proteases, the genus "*Bacillus*" plays a significant role. They are extensively spread in soil and water, and certain

strains can withstand extremely arid or alkaline circumstances. It has been documented that alkaline protease-producing *Bacillus spp*. can be isolated from a several places, including soils with high pH levels and detergent pollution, dried fish, sand soil, milk processing plants, and slaughter houses. Detergent proteases, the majority of which are alkaline serine proteases active in the neutral to alkaline pH range, account for the most significant portion of the enzyme market (Sharma *et al*., 2017).

It has been demonstrated that *Bacillus* alkaline proteases can be used as a dehairing agent, showing elastolytic, keratinolytic, and mild hydrolytic capabilities. Due to their capacity to release significant volumes of highly active enzymes, *Bacillus* strains are recognised as significant sources of commercial alkaline protease (Sharma *et al*., 2015).

To determine the practical relevance of alkaline proteases isolated from various microbial species, a great deal of research has been done on these enzymes. A comprehensive understanding of their precise industrial application can be gained by a detailed analysis of its properties, including the ideal pH, temperature, effects of solubilizing agents, and inhibitory variables. They have a reputation for thriving in extreme salinity and high alkaline pH (Joshi *et al*., 2008).

One of the most significant classes of industrial enzymes, alkaline proteases, account for almost 60% of all enzyme sales globally (Kembhavi *et al*., 1993). Applications for alkaline protease span a variety of industries, such as the detergent business, silver recovery from X-ray films, bleaching agents, hair removers in the leather industry, and protein degradation of shrimp waste to make chitin, among others (Nilegaonkar *et al*., 2007 and Shikha *et al*., 2007). The main expense in the entire process—roughly 30–40% of the total production cost—lies in the growth substrates used in the manufacture of these enzymes. So, this issue necessitates for an economical growing medium that can significantly reduce the costs associated with this enzyme production (Chandran *et al*., 2014).

TABLE 1

VARIOUS MICROBIAL SOURCES AND THEIR APPLICATIONS

Microbial source	**Applications**
Bacillus lentus	Detergent, food, silk degumming
Bacillus licheniformis	Detergent, silk degumming, Feed, Denature cleaners
Bacillus alcalophilus	Detergent
*Bacillus sp.*Tk1 and Tk2	Detergent, food and pharmaceutical
Bacillus subtilis	Cosmetics, pharmaceuticals and detergent
Bacillus pumilus Cbs	Detergents, dehairing
Bacillus cereus	Detergents and removal of blood stains
Streptomyces sp.	Dehairing, feather hydrosylation
Pseudomonas aeruginosa PD	Collagen replacement therapy, waste treatment, removal of blood stains and dehairing
Bacillus licheniformis RP1	Chitin extraction, chicken feather degradation and dehairing
Aspergillus flavus	Detergent industry, peptide synthesis
Pseudomonas fluorescens	Detergent and textile industry
*Thermoactinomyces sp.*RM4	Leather industry for dehairing process
*Bacillus sp.*K-30	Deprotenisation of rice bran and detergents
Bacillus subtilis PE-11	Detergents
Bacillus clausii	Industrial sector
*Bacillus sp.*SSR1	Detergents
Engyodontium album BTMFS10	Detergents and silver recovery

Source: Gupta *et al*., 2002

2.4 SELECTION OF MICROORGANISMS AND MAINTENANCE

The numerous bacterial strains that are known to produce alkaline proteases were first isolated from their natural surroundings. They were sub cultured in vitro using the appropriate medium equipment (Masi *et al*., 2018). Following identifying of the potential strains from the previous colonies, their enzyme activity was revealed when introduced to the substrates made up of proteins (Patel *et al*., 2006). The isolated strains were then studied for their kinetics and identified the best circumstances for their most active and rapid growth.

2.5 PROTEASE PRODUCTION SYSTEMS

Microbial protease is commercially manufactured through submerged or solid-state fermentation systems. Both technologies can be employed for production depending on the quantities of product needed and the availability of the necessary materials. Both systems are beneficial, but choosing between them has pros and cons (Jessika *et al*., 2019 and Oskouie *et al*., 2008).

2.5.1 SUBMERGED FERMENTATION TECHNIQUE

To produce enzymes, submerged fermentation is often carried out utilising batch or fed -batch methods in an aerobic stirred tank reactor (Chandran *et al*., 2014). The advantages of submerged fermentation include greater chances for process control and analysis and the foundation for pre-planned research to boost fermentation yield using an optimised medium (Saranraj *et al*., 2017).

TABLE 2

PROTEOLYTIC ENZYMES (ALKALINE PROTEASE) FROM BACTERIA BY SUBMERGED FERMENTATION

Microorganism	**Substrate**	**Proteolytic production**	**References**
Bacillus sp.K -3	Casein	17.4 U/mL	Yin *et al*., 2019
Bacillus sp.HL-8	Glucose	30.8 U/ mL	Brittney *et al*., 2019
Bacillus–firmus 7728	Casein	215 U/mL	Rao and Narasu,2007
Bacillus lichenformis	Casein	102 U/mg	Potumarthi *et al*., 2007
Bacillus cereus	Fructose and peptone	120 U/mL	Joshi *et al*., 2007
Bacillus sp.HL-4	Date waste	57,420 U/mL	Darani *et al*., 2008
Bacillus circulans	Wheat bran, rice bran	808 U/mL	Jaswal *et al*., 2008
Bacillus sp.HL.9	Glucose and soybean	338U/mL	Saxena and Singh , 2010
Bacillus sp.HS-4	Glucose, casein and gelatin	250 U/mL	Sehar and Hameed , 2011
Bacillus sp.N-40	Fructose, skim milk	338 U/mL	Sevinc and Demirkan , 2011
B.lincheniformis	Wheat flour, rice flour	10,738 U/mg	Akecan., 2012
Pseudomonas k-9	Wheat bran	60.5U/mL	Radha *et al*., 2014

Source: Gemechu *et al*., 2020

2.5.2 CRUDE PROTEASE PREPARATION AND ITS PURIFICATION TECHNIQUES

Purification is a multi-step procedure that uses numerous ways to obtain pure enzymes. There are many different approaches, and the one used depends on the source of the enzyme, its stage of function, and whether it is stable or not. The cost of production and the benefit should be considered when isolating enzymes on a big scale for commercial purposes (Chandran *et al*., 2018). Economically viable enzyme extraction and purification techniques should be used. Based on these prerequisites, the following techniques are more effective for extracellular protease extraction and purification (Saranraj *et al*., 2017).

2.5.3 CONCENTRATION

Separating water from the raw enzyme is the primary goal of downstream enzyme processing. This stage is the most crucial step since fewer enzymes are present in the cell-free filtrate as the enzyme concentration increases. At the moment, membrane separation techniques, particularly ultrafiltration, are widely used to recover an enzyme from evaporation. It is affordable, doesn't interfere with the functioning of enzymes, and provides infiltration for salt removal and adjusting salt concentration (Chandran *et al*., 2018 and LidijaIzrael-Zivkovic *et al*., 2010).

2.6 THE EFFECT OF PRODUCTION PARAMETERS ON THE ACTIVITY AND STABILITY OF ALKALINE PROTEASE

2.6.1 THE EFFECT OF pH

Alkaliphilic microorganisms, or that favour an alkaline environment, rely heavily on the extracellular pH for the growth of their cells, their survival, and the creation of enzymes. Alkaliphilic microorganisms typically prefer a pH of around 10 for quick growth and high production yield. For calculating the intracellular pH of these microorganisms, two techniques are helpful. The first technique involves determining the intracellular enzymes to estimate the ideal pH. This approach is recommended as the most effective for intracellular bacteria and other microbes. The second approach is predicated on weak bases that are neither carried by cells nor stored by them. The distribution of these bases is measured using this method (Pant *et al*., 2015).

The ideal pH for the formation of alkaline proteases often falls between 9 and 11, with 10 being the most common value. This category includes many alkaline protease-producing bacterial and fungal species such as *Aspergillus clavatus* (pH 9.5), *Bacillus firmus* 7728 (pH 9), *Bacillus sp*.JB-99 (pH 11), *Bacillus sp*.SSR1 (pH 10), *Bacillus firmus* 7728 (pH 9), and *Penicillium sp*. (pH 9). It is unknown how pH affects bacterial metabolism in culture broth at the molecular level. Because the medium's pH affects the proton motive force during chemiosmosis, great metabolic efficiency is feasible when optimal pH ranges. Consequently, pH is a crucial component that needs to be optimised (Jignasha *et al*., 2007 and Smita *et al*., 2012).

2.6.2 THE EFFECT OF TEMPERATURE

Temperature is the second most crucial factor that needs to be regulated to achieve the highest protease yield and cell development. Controlling and improving this aspect is crucial for the reason mentioned above. Most protease-producing bacteria perform best at temperatures between 50 and 70°C.

Some of the microbes that like temperatures between 50°C and 70°C include *Micrococcus sp*. (50°C), *Actinomycete* MA1-1 (50°C), *Pseudomonas aeruginosa* MN1 (60°C), *Bacillus clausii* I 52 (60°C), *Aspergillus oryzae* CH93 (50°C). Although the majority of protease-producing bacteria are found in this temperature range, there are some exceptions. Some of these microorganisms include *Aspergillus niger* (30°C), *Bacillus amovivorus* (37°C) and *Aspergillus nidulans* HA-10 (35°C). When crude protease is incubated at various temperatures, its activity and stability can be assessed (Chandran *et al*., 2018 and Jayati *et al*., 2006). The optimum temperature for a crude protease is the one at which it exhibits the most significant activity and stability.

TABLE 3

PROCESS OPTIMIZATION OF ALKALINE PROTEASE USING VARIOUS BACTERIAL SPECIES

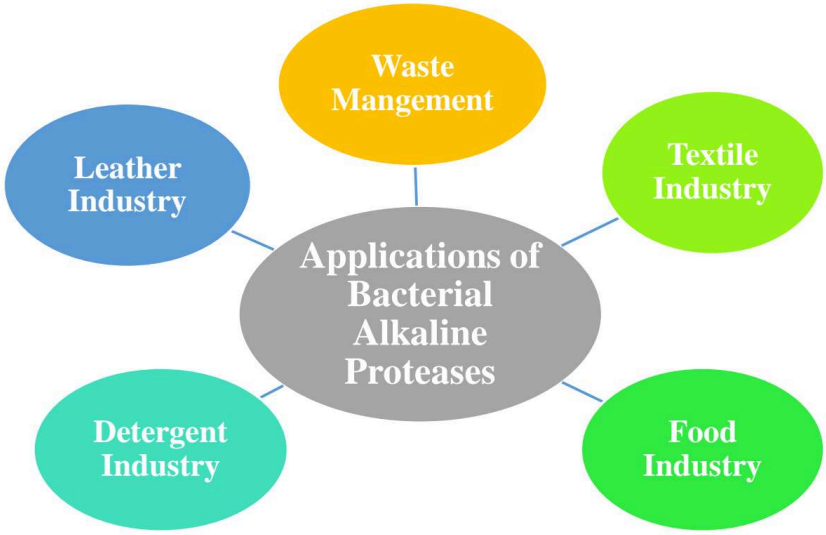
Bacterial species	**Incubation period (Hours)**	**Optimum temperature °C**	**Optimum pH**	**Substrates**	**References**
Bacillus cereus	24	57	8.5	Casein	Han-seungjoo and Chang 2004
Bacillus licheniformis	48	55	7.8	Casein	Gupta *et al.*,2002
Bacillus clausii	36	37	11	Casein	Patel *et al.*,2005
Bacillus mojavensis	24	50	7.5	Casein	Ashi *et al.*,2008
Bacillus pantotheneticus	48	47	8.5	Casein	Varela *et al.*,2002
Bacillus sphaericus	36	50	10.5	Azocasein	Kunamneni *et al.*, 2005
Bacillus subtilis	24	35	8.5	Casein	Feng *et al.*,2001
Bacillus pumilus	48	40	8.8	Casein	Folasade *et al* ., 2005
Bacillus orikoshu	96	47	9	Casein	Qasim *et al.*,2003

Source: (Masi *et al.*, 2014)

2. 7 INDUSTRIAL APPLICATIONS OF MICROBIAL ALKALINE PROTEASES

Proteases perform a wide range of tasks, from the cellular to the organ and organism levels, to create cascade systems like hemostasis and inflammation. Their participation in the life cycle of pathogenic organisms has made them a potential target for the development of therapeutic medicines for deadly diseases, including cancer and AIDS. Microbial proteases are used more frequently to treat various diseases, including cancer, inflammation, cardiovascular problems, necrotic wounds, etc. Proteases are immunostimulating substances; when a protease was used concurrently with an antibiotic, the antibiotic concentration increased at the target location.

FIGURE 3: APPLICATIONS OF BACTERIAL ALKALINE PROTEASES



Source: Gemechu *et al*., 2020

2.7.1 LEATHER INDUSTRY

Numerous processes are involved in processing leather, including soaking, dehairing, batting, and tanning. The traditional methods of preparing leather include dangerous chemicals like sodium sulfide, which pose issues with pollution and wastewater disposal (George *et al*., 2014). Enzymes have been used successfully in place of chemicals to improve leather quality and lessen environmental impact (Jisha *et al*., 2013).

Protease is one of several types of enzyme that is quite helpful since it may dissolve the peptide bond found in hair (Warabo, 2014). Proteolytic enzymes produced by *Bacillus* species may replace chemical agents used in the leather industry, which could have significant economic and environmental effects. It has both economic benefits and environmental contributions. On the one hand, it is inexpensive since it can be made in vast quantities from inexpensive and waste components. On the other side, because it doesn't contain any hazardous compounds, it is environmentally safe (Gupta *et al*., 2002 and Sundararajan *et al*., 2011).

2.7.2 DETERGENT INDUSTRY

Proteases are one of the most common additions in all detergents, including those used in homes as cleaning agents and denatures. Around 25% of all enzyme sales globally are attributable to the use of proteases as additives in cleaning businesses. The biological and environmental friendly detergent was initially offered in 1956 by BIO-40 Trading Corporation. This bio detergent, also known as bacterial enzyme detergent, mostly comprises bacterial enzymes (Ray, 2012).

Protease's pI is crucial to detergent effectiveness (ionic strength). Protease is better suited for this purpose if its pI matches the pH of the detergent solution, as it is self-evident. In addition to the purposes listed above, adding protease to detergent formulation dramatically improves the cleaning of wastes with high protein content. It provides special advantages that cannot be attained with traditional detergent methods (AlyaSellami-Kamoun *et al*., 2008). Around 50% of all detergents contain enzymes, making their usage in detergent formulations routine in developed nations. Most of these enzymes are produced by *Bacillus* species (Kumar *et al*., 2008).

Enzymes have highly practical and significant applications in the detergent and textile sectors. Also, it offers a solid alternative to chemical detergent. Detergent protease currently accounts for 30% of the global enzyme market. Proteases are increasingly replacing minor additions as the main constituents in detergents. Protease can be used as a detergent additive due to the following characteristics: activity and stability at alkaline pH, stability at high temperatures, and compatibility with other chelating and oxidising agents (Rathakrishnan *et al*., 2011).

The following characteristics can be used to identify good detergent proteases: It is most active at alkaline pH values between 9 and 11, very active at low wash temperatures between 30 °C and 40 °C, and stable even at high temperatures of about 60 °C. It is also stable when used with other detergent ingredients and inhibitors and stored for a long time. It has broad specificity once more, encompassing all proteins from all sections. All of this protein may be broken down by this enzyme. Alkaline proteases are beneficial for industrial and commercial applications because they have suitable qualities, including high stability and activity under hard conditions, such as high temperature and pH (Karatas *et al*., 2013). Adding cellulose, lipase, and amylase to washing detergent is anticipated to improve the protease performance (Kumar *et al*., 2008).

2.7.3 SILVER RECOVERY

In the bioprocessing of used X-ray or photographic films for silver recovery, alkaline proteases are crucial. These waste films can serve as a reliable supply of silver for a range of uses since their gelatin layer contains 1.5–2.0% silver by weight. Traditionally, this silver was collected by burning the films, which pollutes the environment. Also, this procedure will not allow for the recovery of the polyester base film. By using proteolytic processes, it is possible to remove the silver from the protein layer encasing this gelatin-bound silver. Gelatin is hydrolyzed using enzymes to extract silver, but the polyester film basis can also be recycled. *Bacillus subtilis* alkaline protease breaks down the gelatin layer in 30 minutes at 50 to 60°C and releases the silver (Gupta *et al.*, 2002).

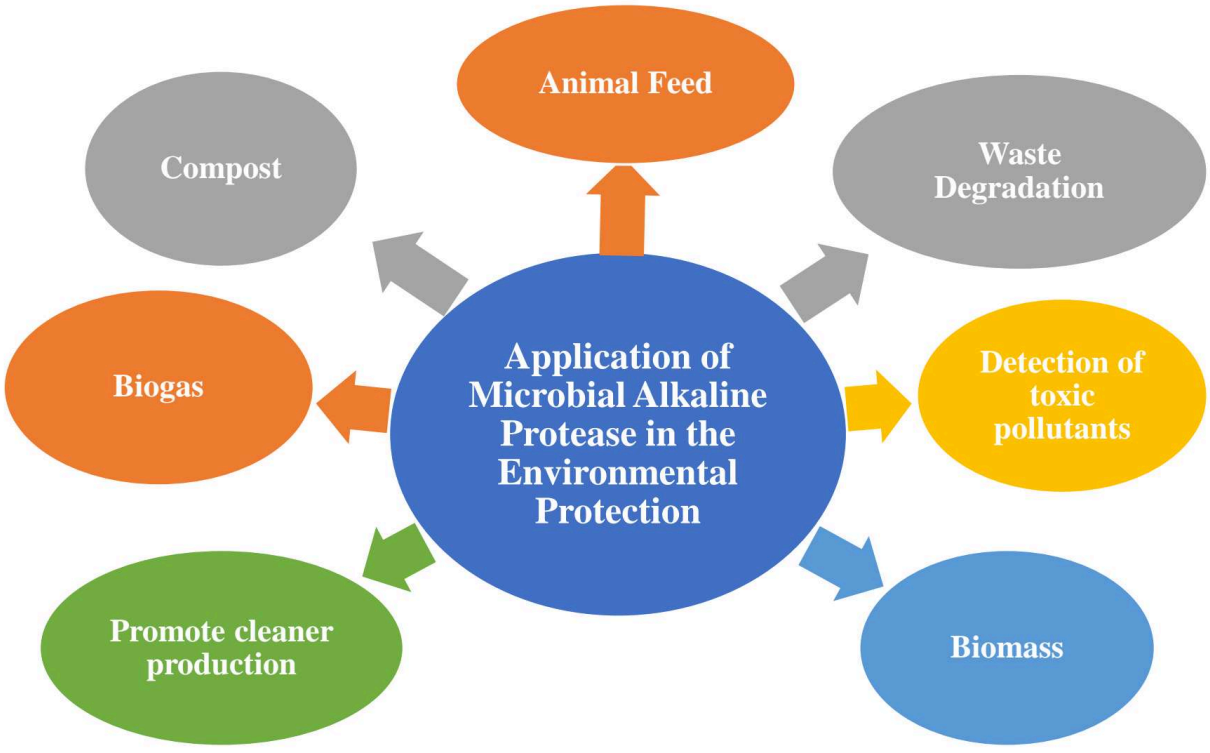
2.7.4 TEXTILE INDUSTRY

Proteases enzymes are used to pulp and thin yarns and improve the shape of yarns and cotton fibre. Protease can also be used in the textile industry to remove the stiff and unappealing gum coating of sericin from the raw silk fibre in order to improve lustre and softness. In finishing wool, proteases are utilised. It aims to increase surface attractiveness, pull-out performance, and comfort (less prickly, higher softness). Another use of degumming silk creates a sand-washed look on silk clothing. It is asserted that treating silk-cellulosic mixes can produce some distinctive results. In order to get rid of the protein gums that are employed to thicken printing pastes, protease is also used to wash down printing screens after usage (Kumar *et al*., 2016).

2.7.5 WASTE TREATMENT

Alkaline protease has many useful uses in organising waste from various food processing businesses and household activities. They have the capacity to dissolve proteins into wastes through multi-step processes to recover liquid or dry solid concentrates with excellent nutritional value for fish or cattle. Enzymes may target specific uncontrollable contaminants and remove them by transforming or precipitating them into the end products. Processing of waste feathers from poultry slaughterhouses involves alkaline proteases from *Bacillus subtilis*. Similar to this, numerous additional keratinolytic alkaline proteases are used in food technology to produce amino acids and peptides for the breakdown of waste keratin in household waste as well as a depilatory agent to eliminate hair in bathtub drains that causes foul odours in homes and public places (Ali *et al*., 2017).

FIGURE 4: APPLICATION OF MICROBIAL ALKALINE PROTEASE IN THE ENVIRONMENTAL PROTECTION



Source: Gemechu *et al*., 2020

2.8 LEMON AND ITS PEELS

A vital member of the Rutaceae family of medicinal plants is the lemon. *Citrus* flavonoids exhibit various biochemical effects, such as antibacterial, antifungal, antidiabetic, anticancer, and antiviral (Burt, 2004 and Ortuno *et al*., 2006). *Citrus* fruit peels are a plentiful supply of volatile oils, coumarins, and flavonoid glycosides (Sultana *et al*., 2007). According to Ahmad *et al.*, (2006), various polymethoxylated flavones exhibit a variety of major bioactivities that are extremely rare in other plants. *Citrus* fruit fibre also includes bioactive substances, such as polyphenols, the most significant of which is vitamin C (ascorbic acid). They undoubtedly prevent and treat vitamin C deficiency, which is the cause of scurvy (Aronson, 2001).

SCIENTIFIC CLASSIFICATION:

Kingdom : Plantae, Angiosperms, Eudicots, Rosids

Order : Sapindales

Family :Rutaceae

Genus : *Citrus*

Species : *C. limon*

Binomial name - *Citrus limon*

Oval citrus fruits called lemons have smooth, permeable skin. While some lemons have a rounded base, others have a pointed tip on the underside of the fruit. Some lemons are quite large and resemble long grapefruits compared to other lemons. Bush lemon, Eureka, Lisbon, Ponderosa, Variegated Pink, Verna, Villafranca, Yen Ben, and Yuzu are just a few of the many lemon types. Lemon fruit can vary in colour from greenish yellow to bright yellow. Lemons and limes have very similar appearances, but lemons are typically more enormous and turn yellow when mature, while limes are green (Mohanapriya *et al*., 2013).

The most popular use of lemon is as a cleaning agent. They can be used to remove physical grime contributions to their high acid content, to lighten stains on cloth, and to mask foul odours due to their potent aroma. These qualities all relate to its use as an energy cleanser. Purification is achieved by adding lemon juice to ceremonial baths, floor washes, or asperging water**.**

Vitamin C, pectin, fibre, and phytochemical compounds with desirable properties, primarily composed of phenolic constituents and essential oils, are abundant in

lemon peels, supporting their possible use in industry. Lemon peels have been found to have many beneficial nutraceutical qualities, including antioxidant, antimicrobial, antidiabetic, anti-obesogenic, lipid-lowering, anti-colitic, anti-arthritic, anti-urolithic, anti-fatigue, and ameliorating muscle atrophy due to inactivity. Lemon peels have many potential uses in the food business, such as 1) the production of food ingredients with beneficial nutritional, antioxidant, and antibacterial qualities and 2) the production of film matrices or active ingredients for food packaging or edible coatings. Additionally, the potential use in aquaculture as healthy fish diet components and in bioremediation as dye and heavy metal removers is encouraging (Jiang *et al*., 2022).

2.9 PAPAYA AND ITS PEELS

The papaya, also known as *Carica papaya* Linn, is a member of the Caricaceae family. The papaya tree grows to 20 to 30 feet and has a solitary stem. The hollow, 8-inch stems range in colour from light green to tawny brown and have noticeable scarring. The fruits are large and oval, and because they have a central seed cavity like a melon, they are sometimes referred to as pepo-like berries. Papaya plants are hermaphroditic or dioecious, and they only produce male, female, or bisexual (hermaphroditic) flowers. Papayas are occasionally "trioecious," meaning that different trees grow either male, female, or bisexual flowers (Arvind *et al*., 2013).

Botanical Classification

Domain : Flowering plant
Kingdom : Plantae
Sub Kingdom : Tracheobionta
Class : Magnoliopsida
Subclass : Dilleniidae
Superdivision : Spermatophyta
Phyllum : Steptophyta
Order : Brassicales
Family : Caricaceae
Genus : Carica

Botanical Name: *Carica papaya Linn*

Papaya skin is frequently used in cosmetic products. Numerous home remedies also employ papaya skin, sunscreen and soothing lotion to aid in rebuilding and repairing injured skin. It is employed as a skin whitening agent, to combat dandruff, as a painkiller, and as a muscle relaxant.

The papaya plant contains the enzyme papain, carotenoids, lycopene, and isothiocyanate, as well as essential minerals, vitamins, and carbohydrates. The cancer-fighting protein papain is abundant in papaya. The fibrin cancer cell wall and protein are broken down into amino acids by papain. Along with papain, it also has lycopene, a pigment that is extremely reactive to free radicals and oxygen. The fermented papaya fruit is a potential antioxidant nutraceutical. As a hydrolase enzyme closely bound to the portion of crude papain that is insoluble in water, papaya lipase is a "naturally immobilised" biocatalyst (Marotta *et al*., 2006).

Papain, veggie pepsin abundant in unripe fruit, is a great digestive aid that facilitates the breakdown of protein in food in acidic, alkaline, and neutral environments. Patients with celiac disease who cannot digest the gliadin protein in wheat can handle it if it is treated with crude papain, and it can tenderise meat. By preparing meat with raw papaya to make it tender and palatable, this information is put to use (Marotta *et al*., 2006).

2.10 SOAP NUT

The Sapindaceae family includes *Sapindus mukorossi*, most commonly known as soapnut. About 6-10% of the weight of the fruits of the *Sapindus* species is made up of saponins. The saponin content of soapnut, which has been used for cleaning reasons for centuries, gives it a detergent-like effect.

It has been discovered that synthetic surface active agents like sodium lauryl sulphate (SLS) have adverse effects on the epidermis, such as irritation and inflammation. There is evidence that soapnut extracts, widely used in home remedies, are suitable for the skin and as a cleansing agent (Bhalekar *et al*., 2017).

It is a common component of ayurvedic preparations like shampoo, cleansers, and medications for treating eczema, psoriasis and removing freckles. It also has a mild insecticidal property and has been used historically for lice removal from the head. The species is widely cultivated in the higher regions of the Indo-Gangetic plains, Shivaliks, and sub-Himalayan tracts between 200 and 1500 metres. It is also known as the Aritha tree or the

soap nut tree, and it is one of the most important trees in Asia's tropical and subtropical regions (Anjali and Divya, 2018).

2.11 *OCIMUM BASILICUM*

Ocimum basilicum L. is the official botanical term for sweet basil. *Ocimum basillicum* belongs to the Lamiaceae family. It stands 50 cm tall, with oval-shaped leaves, purple or white flowers, and a unique perfume. Basil has a long history and is mostly employed as a medicinal element in Unani and Ayurvedic medicine (Muralidharan *et al*., 2004).

Traditional medicine, the manufacture of essential oils, food use, and adding value to summertime beverages are just a few of the benefits for basil (Sajjadi *et al*., 2010). Linalool and methyl- chaviol are the most significant and prevalent chemicals in *Ocimum basillicum,* with cineol in smaller amount. The chemical composition of *Ocimum sp*. is diverse. 0.20–1% of the essential oil from *Ocimum basilicum*'s dry leaf is present. Basil essential oil's chemical makeup changes greatly depending on the season, growing area, and growth stage. However, it mainly contains eucalyptol, linalool, eugenol, methyl-chavicol, geraniol, methyl cinnamate, τ-cadinol, camphor, and carvacrol, as well as aromatic oxygenated monoterpenes (Kathirvel *et al*., 2012 and Elgndi *et al*., 2017).

Experimental Procedure

3.0 EXPERIMENTAL PROCEDURE

It is well known that microorganisms are essential for the industrial creation of intracellular and extracellular enzymes. *Bacillus* species primarily produce extracellular proteases, and industrial sectors frequently use *Bacillus subtilis* to create a variety of enzymes. The primary enzymes made by microorganisms, primarily found in soil and referred to as Hay *Bacillus* and Grass *Bacillus*, are known as proteases.

The worldwide industrial market for alkaline proteases accounts for 60–65% of the total. Proteases are the one class of enzymes most commonly used in the detergent, pharmaceutical, leather, food, and agricultural sectors (Pant *et al*., 2015). Fruits are the most consumed food in the world due to their high nutritional worth and abundance of healthy antioxidants, minerals, vitamins, and fibre. How to fully utilize the waste material is one of the major issues that face the food business globally (Sharmin *et al*., 2016). Fruit by-products can be used in a number of products to add value and are excellent sources of bioactive chemicals.

Lemon peels are a degreaser and a cleaner of mineral deposits because they are made from lemons that contain citric acid. Pectin, a naturally occurring thickening substance that gives the detergent a gel texture, is present in the white pith. For two reasons, limonene is utilized in cleaning products: it has a nice scent and works as a solvent to dissolve grime (Etta-francis *et al*., 2022). Papain, a protease enzyme that clears stains, is present in papaya peels. Additionally, beta-carotene is present, which improves the product's colour. Malic acid, which also functions as a surfactant, is also present in papaya skins (Garcia-Villegas *et al*., 2022). While adding *Ocimum basilicum* essential oils to a product imparts fragrance while preventing many respiratory illnesses like asthma, bronchitis, influenza, and other respiratory conditions. Sweet basil, commonly referred to as *Ocimum basilicum*, is a traditional medicinal plant (Shahrajabian *et al*., 2020).

Protease enzymes from *Bacillus subtilis* are isolated via purification techniques based on a number of factors, including solubility (precipitation with salts) and size (dialysis) (Castillo-Yaez *et al*., 2004).

The experimental designs of the present study entitled "**Development of eco friendly liquid laundry detergent with fruit peels and protease from *Bacillus subtilis,"***

are as follows:

3.1 Microorganism Collection and Culture Condition

3.2 Preparation of inoculums

3.3 Substrate screening (qualitative assay of protease)

3.4 Shake flask fermentation with the selected medium

3.5 Effect of Carbon and Nitrogen Sources on Protease Production

3.6 Effect of Incubation Time on Protease Production

3.7 Precipitation of Protease - Ammonium Sulphate Precipitation

3.8 Purification of Isolated Protease

3.8.1 Dialysis

3.9 Study of Purification Profile

3.9.1 Determination of Total Protein

3.9.2. Assay of Protease Activity

3.9.3 Specific Activity

3.9.4 Recovery Percentage

3.9.5 Purification Fold

3.10 Characterization of Protease

3.10.1 Determination of Optimum pH

3.10.2 Determination of Optimum Temperature

3.10.3 Stability of protease

3.10.3.1 Stability for pH

3.10.3.2 Stability for Temperature

3.11 Eco friendly liquid laundry detergents and its analysis

3.11.1 Extraction of Lemon Peels

3.1 MICROORGANISM COLLECTION AND CULTURE CONDITION

Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore (Tamil Nadu), India, provided the *Bacillus subtilis* and *Candida albicans* was used in the present study. *Bacillus subtilis* strain was kept at 4°C and grown on nutrient agar slants. *Candida albicans* was grown on YEPD media in plates and incubated at 37°C.

3.2 PREPARATION OF INOCULUMS

A 50 ml nutrient broth (g/L) was inoculated with a loopful culture of the *Bacillus subtilis* strain. The culture was incubated at 37°C for 72 hours while being shaken (150 rpm). The inoculums were 1 millilitre of this broth culture. Appendix I outlines the process in depth.

3.3 SUBSTRATE SCREENING (QUALITATIVE ASSAY OF PROTEASE)

Three distinct substrates (Gelatin, Casein, and Skim milk) were used for the substrate screening, carried out in a nutrient agar medium and maintained at 37°C. Appendix II and Appendix III provides a detailed explanation for this experiment.

3.4 SHAKE FLASK FERMENTATION WITH SELECTED MEDIUM

Since shake flasks are simple and may be used simultaneously, they are frequently used for medium optimization. Shake flasks have a limited oxygen supply in comparison to the production process, which is typically carried out in stirred-tank bioreactors, and little control over crucial process variables like pH (Link and Botz ., 2011)

4% inoculums were added to a pH 8.0 optimized production medium, then incubated with constant shaking to produce alkaline protease. The procedure for the same is explained in Appendix IV.

3.5 EFFECT OF CARBON AND NITROGEN SOURCES ON PROTEASE PRODUCTION

In place of dextrose, simple and complex carbon sources (sucrose, galactose, and starch) as well as in place of yeast extract, nitrogen sources (peptone, ammonium nitrate, and ammonium chloride) were used to test the effects of carbon and nitrogen sources on protease production.

To substitute carbon and nitrogen sources, respectively, each source was used at concentrations of 1% (w/v) and 0.55% (w/v). The protease yield was evaluated after 72 hours of incubation at 37°C with shaking at 100-150 rpm. Following production, a quantitative yield estimate was determined.

3.6 EFFECT OF INCUBATION TIME ON PROTEASE PRODUCTION

Three 100-mL conical flasks of the optimised protease production medium were made, into which 1% *B. subtilis* culture broth was added and kept for shaker fermentation. Each beaker was centrifuged at 5000 rpm for 30 minutes after being filtered every 24 hours. Protease was measured using quantitative techniques using the produced culture filtrate solution (Pant *et al*., 2015).

3.7 PRECIPITATION OF PROTEASE - AMMONIUM SULPHATE PRECIPITATION

The initial purifying and concentration step is frequently, ammonium sulphate precipitation. It is founded on the salting-out theory. For the fractional precipitation of

proteins, ammonium sulphate is a salt that is especially helpful. It is inexpensive, readily soluble, readily available in a highly purified form, and allows for substantial changes in ionic strength.

A solid substance or a known-saturation solution, typically a completely saturated (100%) solution, can be added to a solution to change its ammonium sulphate concentration (Sadasivam and Manickam., 2004). With various amounts of ammonium sulphate (0-20, 20-40, 40-60, 60-80, and 80-100%), protease was precipitated from the crude homogenate. The process is outlined in Appendix V.

3.8 PURIFICATION OF ISOLATED PROTEASE

From the precipitation mentioned above, the sample showing the highest specific activity were taken for further purification studies.

3.8.1 DIALYSIS

Salts or other small substances can be removed from a solution using dialysis (Berg, 2002). To help with the desalting of the proteins, the portion of the ammonium sulphate precipitated sample with the greatest specific activity was purified by dialysis using 20 mM Tris-HCl buffer. The procedure for the same is explained in Appendix VI.

3.9 STUDY OF PURIFICATION PROFILE

Total protein content, protease activity, specific activity, recovery percentage and purification fold of the ammonium sulphate precipitated and dialyzed samples were determined.

3.9.1 DETERMINATION OF TOTAL PROTEIN

Precise quantification can be obtained by hydrolyzing the protein and determining the amino acids alone. The Lowry *et al*. (1951) method is widely used because it is sensitive enough to provide a moderately constant number. This technique typically determines the enzyme extract's protein content (Sadasivam and Manickam., 2004). Using Lowry's technique, the samples' total protein content was determined; the process is detailed in Appendix VII.

3.9.2. ASSAY OF PROTEASE ACTIVITY

The protease activities of the samples were determined by protease assay. The procedure for the same is given in Appendix VIII.

3.9.3 SPECIFIC ACTIVITY

The specific activity of the isolated protease samples was calculated as follows:

$$\text{Specific Activity or Units/mg protein} = \frac{\text{Units /ml enzyme}}{\text{mg protein/ml enzyme}}$$

3.9.4 RECOVERY PERCENTAGE

The recovery percentage of the isolated protease samples was calculated as follows:

$$\text{Recovery percentage} = \frac{\text{Total protease activity of the sample (U/ml)}}{\text{Total protease activity of the crude extract (U/ml)}} \times 100$$

3.9.5 PURIFICATION FOLD

The purification fold of the isolated protease samples was calculated as follows:

$$\text{Purification fold} = \frac{\text{Specific activity of the sample}}{\text{Specific activity of the crude extract}}$$

3.10 CHARACTERIZATION OF PROTEASE

The partially purified - ammonium sulphate precipitated protease was taken for characterization studies.

3.10.1 DETERMINATION OF OPTIMUM pH

The activity of the enzyme on the substrate casein prepared in buffers with various pH ranges between 6 and 10 was examined to determine the optimal pH for the purified protease samples. It will be assumed that the enzyme activity is 100% relatively active at the pH where it records the highest value. In Appendix IX and X, the procedure for this is described in detail.

3.10.2 DETERMINATION OF OPTIMUM TEMPERATURE

After incubating the reaction mixture at various temperatures ranging from 20°C to 60°C, as shown in Appendix XI, the activity of the enzyme was observed to identify the enzyme's optimal operating temperature for the activity of the purified protease enzymes.

3.10.3 STABILITY OF PROTEASE

To determine the enzyme's shelf life, finding the protease's stability for time is crucial.

3.10.3.1 STABILITY FOR pH

After incubating the reaction mixture at varying periods (15 mins, 20 mins, 30 mins, 45 mins and 60 mins) the enzyme activity was observed to identify the enzyme's stability for the optimum pH. The protease activities of the samples were determined by protease assay. The procedure for the same is given in Appendix VIII.

3.10.3.2 STABILITY FOR TEMPERATURE

After incubating the reaction mixture at varying periods (15 mins, 20 mins, 30 mins, 45 mins and 60 mins) the enzyme activity was observed to identify the enzyme's stability for the optimum temperature. The protease activities of the samples were determined by protease assay. The procedure for the same is given in Appendix VIII.

3.11 ECO FRIENDLY LIQUID LAUNDRY DETERGENTS AND ITS ANALYSIS

3.11.1 EXTRACTION OF LEMON PEELS

A method for extracting limonene from the lemon peels is described briefly in Appendix XII.

3.11.2 EXTRACTION OF PAPAYA PEELS

The procedure for extracting bio extract from papaya peels is briefly explained in Appendix XIII.

3.11.3 PRODUCTION OF SOAP NUT LIQUID

A method for the production of soap nut liquid is described briefly in Appendix XIV.

3.11.4 EXTRACTION OF ESSENTIAL OIL FROM *OCIMUM BASILICUM*

The leaves of the *Ocimum basilicum* were dried in a shed place. The essential oil can be obtained either by the soxhlet method of extraction (Mindaryani *et al*., 2007) or by the homemade process.

3.11.5 QUALITATIVE ANALYSIS (PROTEASE ASSAY)

The protease activity of the samples was determined by protease assay. The procedure for the same is given in Appendix III.

3.11.6 pH OF LIQUID LAUNDRY DETERGENT

pH of liquid laundry detergent was tested using pH meter.

3.11.7 FOAM TEST

A Foam test is generally done to test the saponin content in the sample. 1ml solution of the sample was diluted with distilled water to 20ml and shaken in a graduated cylinder for 15 minutes. The development of stable foam suggests the presence of saponins.

3.11.8 ANTIFUNGAL ACTIVITY (AGAR – WELL DIFFUSION METHOD)

The test samples' antifungal activity can pass into the medium and interact with newly seeded test organisms in a plate. A confluent lawn of growth will produce consistently round zones of inhibition. The diameter of the zone of inhibition can be measured in millimetres. *Candida albicans* is used as a test organism, and Amphotericin B is used as the positive control. The experiment was done by using the Agar well diffusion method.

3.12 APPLICATION STUDIES

Cotton fabrics are used for testing the samples. For testing the samples for their activity, factors are taken into note (i) Time and (ii) Temperature.

3.12.1 ACTION ON COAGULATED EGG WHITE

Proteases have the ability to hydrolyze coagulated egg white. The partially purified enzymes were incubated with coagulated egg albumin, and the changes were observed. The procedure for this is given in Appendix XV.

3.12.2 ACTION ON SOYA STAIN

The destaining activity of various test samples was examined with a piece of soya-stained cloth, which was incubated at varying temperatures. The detailed procedure is recorded in Appendix XVI.

3.12.3 ACTION ON METHYLENE BLUE DYE

The destaining activity of the developed liquid laundry detergent was examined with a piece of methylene blue dye cloth, which was incubated at 37°C with respect to time (0min, 5mins, 10mins, and 15mins).

Results & Discussion

4. RESULTS AND DISCUSSION

Almost every cellular process in living beings requires the use of enzymes. In meat tenderization, enzymes break down proteins into smaller molecules to make the meat easier to chew or bite (Prescher and Bertozzi, 2005). Enzymes also help to remove starch or fat stains from garments. Because of its capacity to produce proteases and its highest degree of proteolytic activity, *Bacillus subtilis* was selected as the biological agent in submerged fermentation (Rathakrishnan *et al*., 2012).

Bacillus strains are considered substantial sources of commercial alkaline protease due to their ability to release sizable amounts of highly active enzymes (Sharma *et al*., 2015). Detergents, food, medications, and leather are just a few industrial items that utilise alkaline proteases (Sundus *et al.,* 2016). Due to its activity and stability at alkaline pH levels, high temperatures, and compatibility with other chelating and oxidising agents, protease can be employed as a detergent additive (Rathakrishnan *et al*., 2011). Good detergent proteases can be distinguished by the qualities listed below: It is particularly active at low wash temperatures between 30 °C and 40 °C, very active at alkaline pH values between 9 and 11, and stable even at high temperatures of roughly 60 °C. Additionally, it maintains its stability when combined with other detergent components, inhibitors, and storage for extended period time (Karatas *et al*., 2013).

The results of the present study on "**Development of eco friendly liquid laundry detergent with fruit peels and protease from *Bacillus subtilis*"** are discussed under the following headings:

4.1 Microorganism Collection and Culture Condition

4.2 Preparation of inoculums

4.3 Substrate screening (qualitative assay of protease)

4.4 Shake flask fermentation with the selected medium

4.5 Effect of Carbon and Nitrogen sources on protease production

4.6 Effect of Incubation time on protease production

4.7 Purification profile of protease

4.7.1 Ammonium sulphate precipitated protease

4.7.2 Dialysed protease

4.8 Characterization of Protease

4.8.1 Determination of Optimum pH

4.8.2 Stability for pH

4.8.3 Determination of Optimum Temperature

4.8.4 Stability for Temperature

4.9 Eco friendly liquid laundry detergents and its analysis

4.9.1 Components used for the development of the liquid laundry detergent

4.9.1.1 Partially Purified Protease

4.9.1.2 Extraction of limonene from lemon peels

4.9.1.3 Extraction of bio extract from papaya peels

4.9.1.4 Production of soap nut liquid

4.9.1.5 Extraction of essential oil from *Ocimum basilicum*

4.9.1.6 Eco friendly liquid laundry detergent

4.9.2 Analysis of liquid laundry detergent

4.9.2.1 pH

4.9.2.2 Qualitative Analysis (Protease Assay)

4.9.2.3 Foam Test

4.9.2.4 Antifungal activity (Agar – well diffusion method)

4.10 Application Studies

4.10.1 Action on Coagulated Egg White

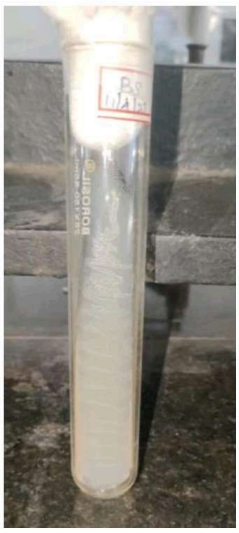
4.10.2 Action on Soya Stain

4.10.3 Action on Methylene Blue Dye

4.1 MICROORGANISM COLLECTION AND CULTURE CONDITION

Figure 5 shows the *Bacillus subtilis* strain cultured on nutrient agar slants. It was then maintained at 4°C.

FIGURE 5: *BACILLUS SUBTILIS* - NUTRIENT AGAR SLANTS



4.2 PREPARATION OF INOCULUMS

FIGURE 6: PREPARATION OF INOCULUMS

Colonies were sub cultured and maintained to obtain a pure culture on nutrient agar plates, as shown in Figure 6.

4.3 SUBSTRATE SCREENING (QUALITATIVE ASSAY OF PROTEASE)

Three distant substrates (Gelatin, Casein and Skimmed Milk) were used for the substrate screening, which was carried out in a nutrient agar medium, as shown in Figure 7 and maintained at 37°C.

FIGURE 7: SUBSTRATE SCREENING

a) Gelatin as Substrate **b) Casein as Substrate** **c) Skimmed Milk as Substrate**

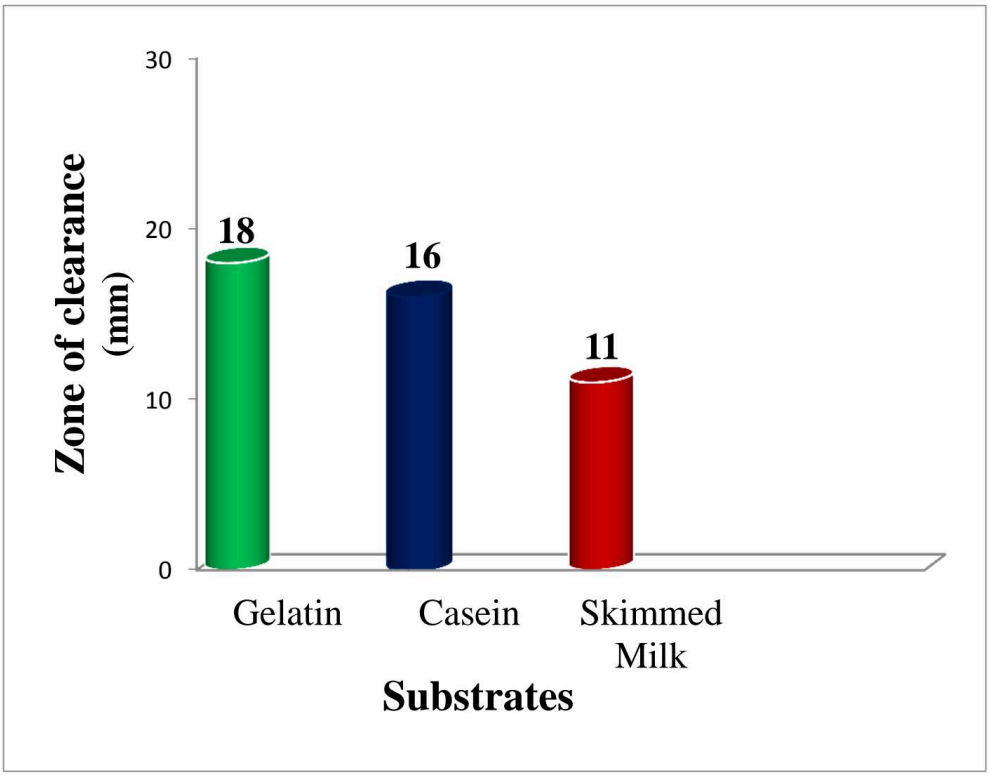
FIGURE 8: HYDROLYSIS (CONTINUOUS STREAKING METHOD)

a) Gelatin Hydrolysis b) Casein Hydrolysis

Figure 8a shows gelatin hydrolysis, where gelatin was used as a substrate, and Figure 8b shows casein hydrolysis, where casein was used as a substrate. From Figure 8,

observations suggest that hydrolysis takes place in both the plates of gelatin and casein as substrate.

FIGURE 9: PROTEOLYTIC ACTIVITY OF *B. SUBTILIS* ON (GELATIN, CASEIN AND SKIMMED MILK AGAR)



Gelatin and Casein were used as the substrate in the nutrient agar medium. The culture was streaked by continuous streaking and incubated at 37°C. After 12 hours of incubation, when suitable indicators were flooded to the plates, hydrolysis took place, which is represented in Figure 9.

The graph was plotted based on the results of Figure 7 (substrate screening). *B.subtilis* had a high proteolytic activity with a clear zone of 18mm when gelatin was used as the matrix, and the zone of clearance for casein and skimmed milk as a substrate was found to be 16mm and 11mm, respectively.

Hence from Figure 9, it was concluded that *Bacillus subtilis* showed higher proteolytic activity on gelatin than on casein or skimmed milk in a nutrient agar medium based on the zone of clearances.

A similar study was reported by Pant *et al.* (2015), where *B.subtilis* had high a

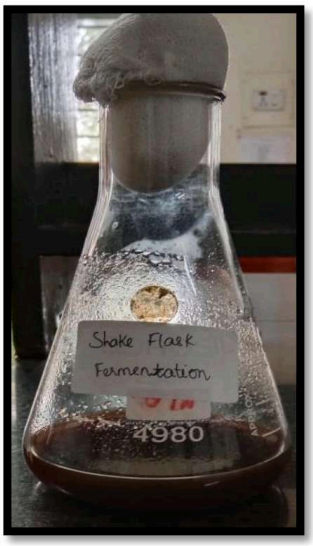
proteolytic activity with a clear zone of 22mm when gelatin was used as the matrix. He also reported that the zone of clearance for casein and skimmed milk as a substrate was found to be 17mm and 15mm, respectively.

Yanga *et al*. (2020) and Karadag *et al*. (2009) stated that the use of casein as the substrate under standard assay conditions gave the highest activity at pH 8.0.

4.4 SHAKE FLASK FERMENTATION WITH THE SELECTED MEDIUM

Figure 10 represents the shake flask fermented culture broth after 72 hours of incubation.

FIGURE 10: SHAKE FLASK FERMENTATION

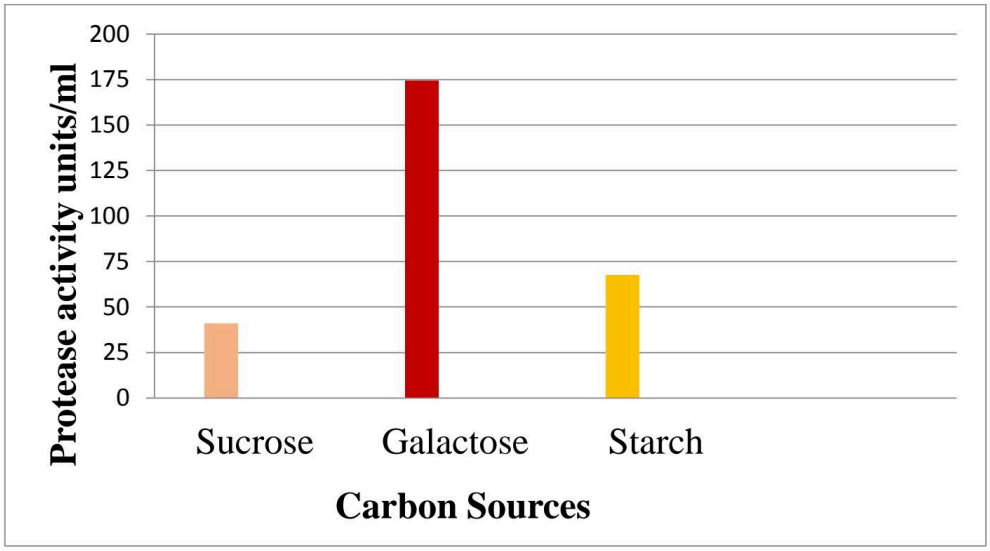


At the end of fermentation, the resultant culture broth was observed with a sweet odour, whereas the odour was absent before fermentation.

4.5 EFFECT OF CARBON AND NITROGEN SOURCES ON PROTEASE PRODUCTION

Figure 11 shows the effect of carbon sources on protease production.

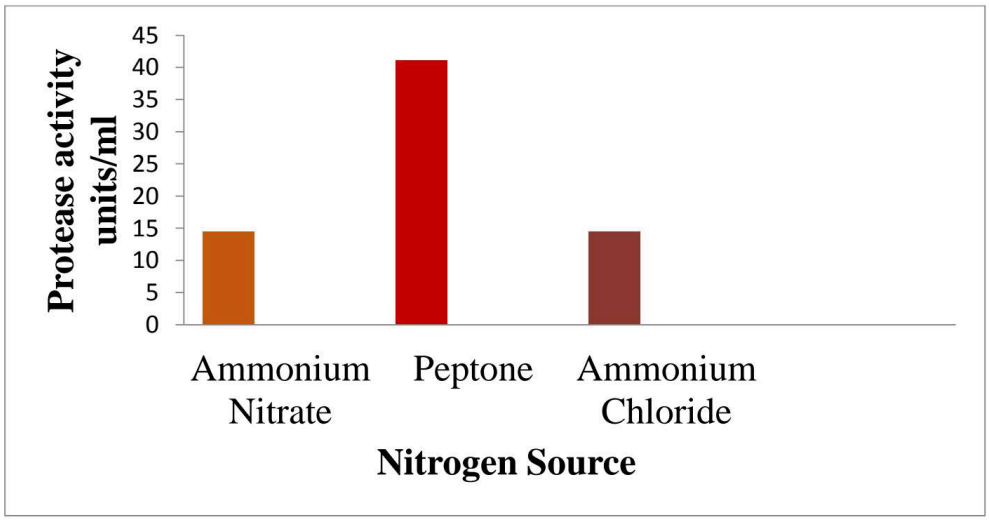
FIGURE 11: EFFECT OF CARBON ON PROTEASE PRODUCTION



From Figure 11, it was observed that the maximum protease activity for the effect of carbon source was found to be galactose with (174.20 U/ml) protease activity, followed by starch (67.69 U/ml), and sucrose showed the least protease activity (41.072 U/ml).

Figure 12 shows the effect of nitrogen sources on protease production.

FIGURE 12: EFFECT OF NITROGEN ON PROTEASE PRODUCTION



From Figure 12, it was observed that the maximum protease activity for the effect of nitrogen source was found to be peptone with (41.072 U/ml) protease activity. Protease activity (14.45U/ml) was found to be the same for ammonium nitrate and ammonium chloride.

It can thus be inferred from Figure 11 and Figure 12 that galactose and peptone had the highest levels of proteolytic activity among the three carbons and nitrogen sources that was examined.

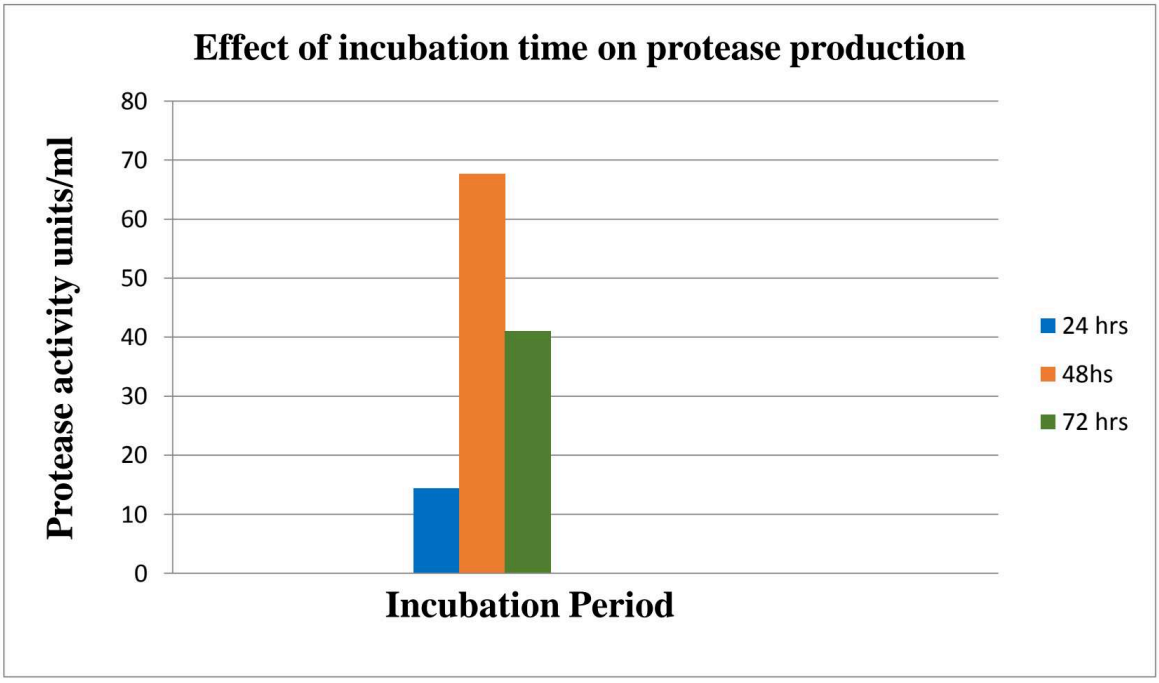
Pant *et al*. (2015) reported that among five carbon and nitrogen sources tested, galactose and peptone resulted in the highest protease activity (236.31 and 175.083 U/mL per min, respectively), while the least enzyme activity was observed with sucrose and ammonium chloride.

A similar studies conducted by Rao *et al.* (2008) and Qureshi *et al.* (2011) revealed that the highest levels of protease production occurred at an incubation temperature of 37°C, a pH of 9.0, and a carbon and nitrogen supply of glucose and sodium nitrate, respectively.

4.6 EFFECT OF INCUBATION TIME ON PROTEASE PRODUCTION

The effect of incubation time was observed for every 24 hrs interval till 72 hrs, and results were depicted in Figure 13.

FIGURE 13: EFFECT OF INCUBATION TIME ON PROTEASE PRODUCTION



From Figure 13, it was observed that the maximum activity was noted at 48hrs of incubation (67.69 U/ml) followed by 72hrs (41.07 U/ml). When incubated only for 24 hrs,

the activity was very less and found to be 14.45U/ml.

Pant *et al*. (2015) reported that the optimum time for protease production was increased gradually from 0 to 36 hours at (243.28 U/mL per min); it then decreased with time. Feng *et al*. (2001) reported that the incubation time for *Bacillus subtilis* was 24 hours.

4.7 PURIFICATION PROFILE OF PROTEASE

4.7.1 AMMONIUM SULPHATE PRECIPITATED PROTEASE

Table 4 shows the effect of various concentrations of ammonium sulphate on the purification profile (protein content, protease activity, specific activity, recovery percentage and purification fold) of protease from *Bacillus subtilis.*

TABLE – 4

PURIFICATION PROFILE OF AMMONIUM SULPHATE PRECIPITATED PROTEASE FROM *BACILLUS SUBTILIS*

Samples	**Protein content (mg/ml)**	**Protease activity (U/ml)**	**Specific activity (U/mg)**	**Recovery %**	**Purification fold**
Crude	0.12	34.54	287.8	100.00	1.00
Ammonium Sulphate Precipitated					
0-20%	0.02	14.48	724	41.92	2.51
20-40%	**0.03**	9.46	315.3	27.38	1.09
40- 60%	**0.03**	**24.51**	817	**70.96**	2.83
60-80%	0.01	9.46	**946**	27.38	**3.28**
80-100%	0.01	4.44	444	12.85	1.54

The following findings can be drawn from Table 4:

The protein content of all the ammonium sulphate precipitated protease samples from *Bacillus subtilis* recorded less concentration when compared to the crude sample (0.12 mg/ml). Among the ammonium sulphate precipitated samples, the highest protein content (0.03 mg/ml) was shown by both 20-40 % and 40-60% ammonium sulphate precipitated samples followed by 0-20% precipitated sample (0.02 mg/ml).

In the case of protease activity, the values for all the ammonium sulphate precipitated samples were lower than that of the crude sample, with the 40-60% precipitated sample exhibiting the maximum activity (24.51 U/ml), followed by 0- 20% ammonium sulphate precipitated sample (14.48 U/ml).

The specific activity of all the ammonium sulphate precipitated samples increased in comparison with crude (287.8 U/mg). The 60-80% ammonium sulphate precipitated sample registered the highest specific activity (946 U/mg), followed by a 40-60% precipitated sample (817 U/mg).

As regards recovery percentage, the 40- 60% ammonium sulphate precipitated sample gave the highest value (70.96%), followed by 0- 20% (41.92%) ammonium sulphate precipitated sample.

The purification fold of the samples also followed a similar trend, with 60-80% ammonium sulphate precipitated sample indicating the highest value (3.28) followed by the 40- 60% ammonium sulphate precipitated sample (2.83).

It can thus be inferred from Table 4 that specific activity and purification fold parameters of the purification profile recorded the highest value for the 60- 80% ammonium sulphate precipitated sample, followed by the 40-60% ammonium sulphate precipitated sample. Hence, the fraction 60-80 % was taken for further purification by dialysis.

According to Pant *et al.* (2015), the protease isolated from B. subtilis is one of the thermally stable enzymes. They also claimed that proteins were recovered from the cell-free culture filtrate by precipitating them with ammonium sulphate at a 75% (w/v) saturation level.

Padmapriya and Williams (2012) suggested in their research that the protease from *Bacillus subtilis* was partially purified by the ammonium sulphate (75%) precipitation method, and the precipitate was used for dialysis.

4.7.2 DIALYSED PROTEASE

Figure 14 represents the samples for 60-80% ammonium sulphate precipitated protease before and after the dialysis sample.

FIGURE 14: AMMONIUM SULPHATE PRECIPITATED PROTEASE BEFORE AND AFTER DIALYSIS

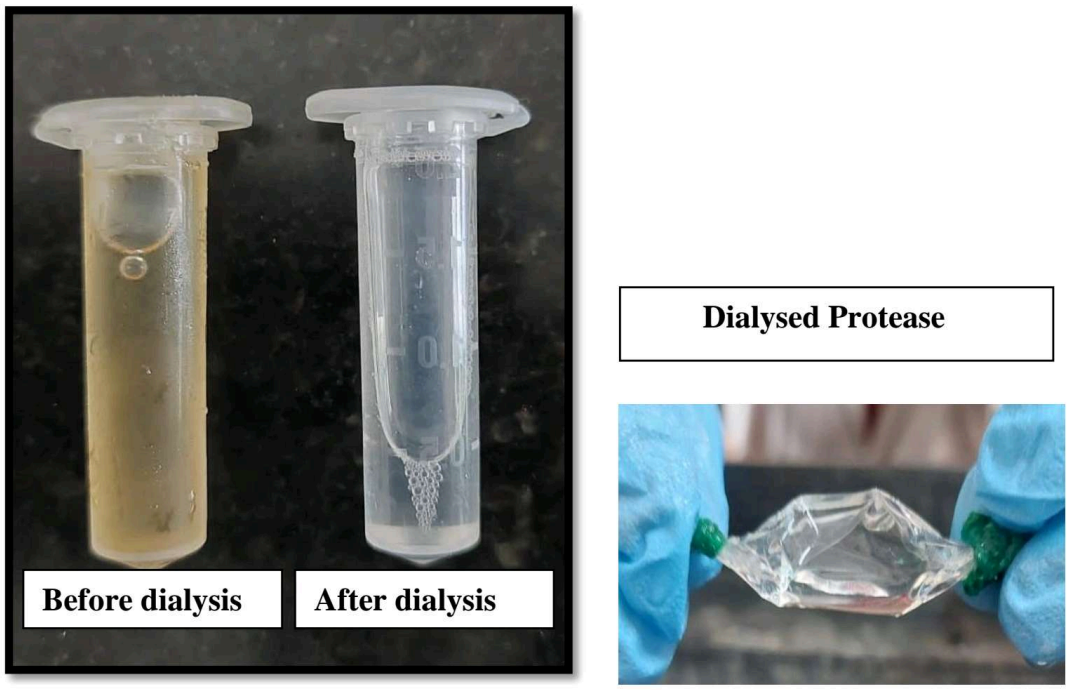


Table 5 records the purification profile of 60- 80% ammonium sulphate precipitated dialysed protease.

TABLE 5

PURIFICATION PROFILE OF DIALYZED PROTEASE

Sample		**Protein content (mg/ml)**	**Protease activity (U/ml)**	**Specific activity (U/mg)**	**Recovery %**	**Purification Fold**
60-80% ammonium sulphate precipitated protease	Before Dialysis	0.01	9.46	946	27.38	3.28
	After Dialysis	0.01	**11.26**	**1126**	**32.59**	**3.91**

It is clear from Table 5 that the protein content of 60-80% ammonium sulphate precipitated protease was found to remain constant after dialysis, whereas the activity of the ammonium sulphate precipitated protease was found to be increased from 9.46 U/ml to 11.26 U/ml. As regards specific activity, the value increased for the sample after dialysis with 60-80% ammonium sulphate precipitated protease exhibiting the maximum value of 1126 U/mg. The recovery percentage of the ammonium sulphate precipitated protease sample also increased (32.59%). On dialysis, the purification fold for the ammonium sulphate precipitated protease sample has increased (3.91) by confirming the purity of the isolated protease.

Thus it can be noted from Table 5 except the protein content, other parameters like protease activity, specific activity, recovery percentage and purification fold were found to be increased after dialysis.

El-Beltagy *et al*. (2005) found that dialysis of alkaline protease crude extract increases the amount of purification.

Padmapriya and Williams. (2012) reported that the neutral protease enzyme was partially purified from *B.subtilis* by using 75% ammonium sulphate precipitation, and their purification fold was 3.12-fold purity, and after dialysis, the purification fold was 5.14-fold purity.

4.8 CHARACTERIZATION OF PROTEASE

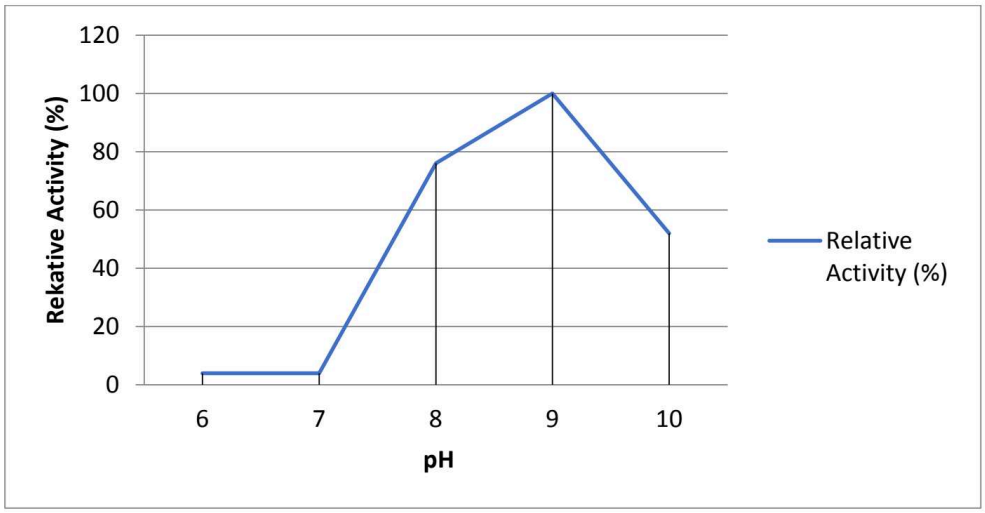
4.8.1 DETERMINATION OF OPTIMUM PH

The effect of varying pH on the relative activity of the partially purified ammonium sulphate precipitated samples is given in Table 6 and Figure 15.

TABLE 6
EFFECT OF pH ON THE ACTIVITY OF AMMONIUM SULPHATE PRECIPITATED PARTIALLY PURIFIED PROTEASE

S.No	pH	Ammonium sulphate precipitated partially purified protease	
		Enzyme activity (U/ml)	Relative activity (%)
1.	6	0.15	4
2.	7	0.15	4
3.	8	2.76	76
4.	9	**3.62**	**100**
5.	10	1.89	52

FIGURE 15: RELATIVE ACTIVITY OF AMMONIUM SULPHATE PRECIPITATED PARTIALLY PURIFIED PROTEASE AGAINST pH



From Table 6, it is seen that 100% relative activity was found at pH 9.0 with enzyme activity 3.62 U/ml, followed by 76% activity found at pH 8.0 with enzyme activity 2.76 U/ml, and 52% activity at pH 10.0 with enzyme activity 1.89 U/ml. Hence it can be said that relative activity increased from pH 6 to 9 and then decreased from pH 10.

Thus from Table 6, it can be inferred that the partially purified proteases have an optimum pH of 9.0 since the relative activity was 100% at that pH.

A similar study was done by Gul *et al.* (2008) and recorded that Bacillus subtilis BS1 showed optimum pH at 9 and displayed 100% relative activity. Naidu (2011) suggested in their study that the protease function was found to be best at pH 9.

4. 8.2 STABILITY FOR pH

The stability of protease is analysed for the optimum pH, recorded and as shown in Table 7.

TABLE 7
STABILITY FOR pH

The pH 9 is considered the optimum pH after observing the results from Table 6.

S.No	**pH 9**	**Enzyme activity (U/ml)**
1.	15mins	0.10
2.	20mins	**0.94**
3.	30mins	0.63
4.	45 mins	0.42
5.	60mins	0.89

From Table 7, it is seen that at pH 9, maximum protease activity (0.94 U/ml) was found to be at 20 minutes of incubation followed by 60 mins of incubation with enzyme activity (0.89 U/ml) and 30 mins of incubation with enzyme activity (0.63 U/ml).

Hence, from Table 7, it can be inferred that partially purified protease has an optimum pH of 9 and is stable for 20mins.

Yossan *et al.* (2006) suggested that the pH stability of protease produced from *B. subtilis* PE-116 and *B. subtilis* CN215 were found to be in the ranges of 8 to 11 and 7 to 11 with the relative activity of more than 90% and 70%, respectively. Gul *et al.* (2008) reported that after 20 minutes of incubation and various pH levels, the enzyme activity of the protease generated by *Bacillus subtilis* BS1 was measured at pH 9, and the enzyme displayed 100% of its maximal activity.

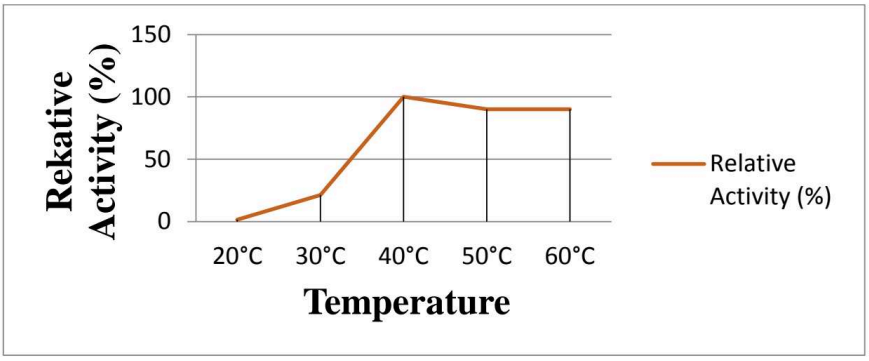
4.8.3 DETERMINATION OF OPTIMUM TEMPERATURE

The effect of temperature on the activity of the Ammonium sulphate precipitated partially purified protease isolated from *Bacillus subtilis* is recorded in Table 8 and Figure 16.

TABLE 8
EFFECT OF TEMPERATURE ON THE ACTIVITY OF AMMONIUM SULPHATE PRECIPITATED PARTIALLY PURIFIED PROTEASE

S.No	Temperature (°C)	Ammonium sulphate precipitated partially purified protease	
		Enzyme activity (U/ml)	Relative activity (%)
1.	20°C	0.07	1.6
2.	30°C	0.94	21.3
3.	40°C	**4.42**	**100**
4.	50°C	3.98	90
5.	60°C	3.98	90

FIGURE 16: RELATIVE ACTIVITY OF AMMONIUM SULPHATE PRECIPITATED PARTIALLY PURIFIED PROTEASE AGAINST TEMPERATURE



Both Table 8 and Figure 16 clearly depicted a gradual increase in the relative activity of the protease from 20°C to 40°C and started to decrease from 50°C to 60°C. The ammonium sulphate precipitated partially purified protease exhibited a relative activity of 100 % at 40°C with enzyme activity (4.42 U/ml) followed by 90% relative activity at 50°C and 60°C with enzyme e activity (3.98 U/ml).

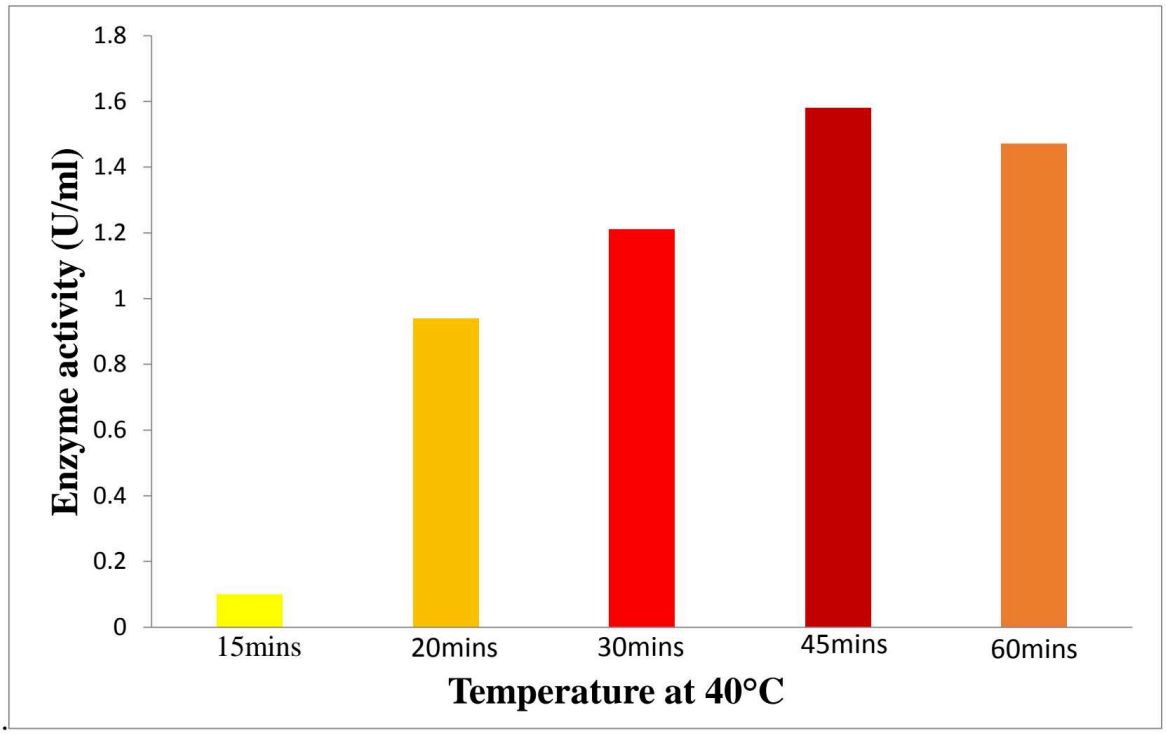
Hence, from Table 8, it can be inferred that the partially purified protease have an optimum temperature of 40 °C with a relative activity of 100%.

A similar study was done by Yossan (2006) and suggested that the protease activity was active at 30 to 50°C but inactive in the ranges of 55 to 90°C. Naidu (2011) also reported in her study that the protease function was observed to be best at 40°C.

4.8.4 STABILITY FOR TEMPERATURE

Stability is analysed by varying the incubation time in protease assay. The stability of protease is analysed for the optimum temperature, as shown in Figure 17.

FIGURE 17: STABILITY FOR TEMPERATURE



From Figure 17, it is seen that the enzyme activity started to increase gradually from 15mins of incubation and reached the maximum protease activity (1.58 U/ml) at 45 minutes and started to decrease from 60mins of incubation with enzyme activity (1.47 U/ml) Hence, it can be inferred that partially purified protease has an optimum temperature of 40°C and is stable for 45mins.

Other tests revealed that the maximum levels of protease production, as reported by Rao *et al*. (2008) and Qureshi *et al*. (2011), occurred at 37 °C, pH 9.0, and with glucose

and sodium nitrate as the carbon and nitrogen sources, respectively. According to Naidu (2011), the protease function was shown to be best at pH 9 and 40°C.

4.9 ECO FRIENDLY LIQUID LAUNDRY DETERGENTS AND ITS ANALYSIS

4.9.1 COMPONENTS USED FOR THE DEVELOPMENT OF THE LIQUID LAUNDRY DETERGENT

4.9.1.1 PARTIALLY PURIFIED PROTEASE

Figure 18 shows the partially purified enzyme from *Bacillus subtilis*

FIGURE 18: PARTIALLY PURIFIED PROTEASE FROM *BACILLUS SUBTILIS*

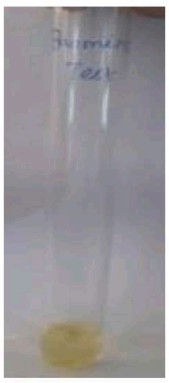
Figure 18 represents the partially purified enzyme from *Bacillus subtilis*. 60-80% ammonium sulphate precipitated protease sample from *Bacillus subtilis* exhibited the best purification profile by means of the highest specific activity and purification fold. The partially purified proteases have an optimum pH of 9.0 at 40°C. 10 parts of partially purified protease were taken for the development of liquid laundry detergent.

The protease has the property to hydrolyse protein; hence it is essentially used in the detergent industry.

4.9.1.2 EXTRACTION OF LIMONENE FROM LEMON PEELS

Figure 19 shows the Extraction of Limonene from Lemon Peels.

FIGURE 19: CONFIRMATION TEST FOR LIMONENE



Bromine Test

A conformation test is used to determine whether limonene was successfully extracted from lemon peels using the methanol extraction procedure. If limonene is present in the extracted sample, the bromine water's colour changes to pale yellow, indicating that the bromine in the solution occupies the space between the limonene's two double bonds.

From the Figure 19 results of the confirmation test for limonene, it was clear that limonene was present in the extracted sample. 1 part of extracted limonene from lemon peels was taken for the development of liquid laundry detergent.

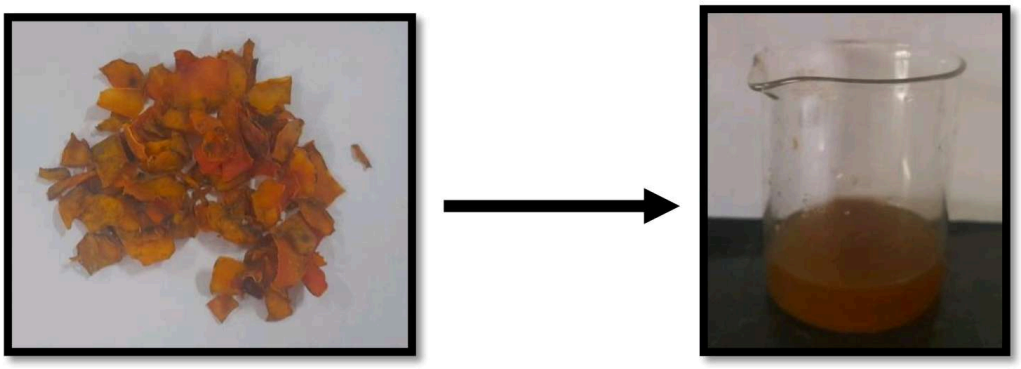
Sikdar and Nikila (2017) reported that citrus oil extracted from lemon peels by cold press method and confirmed the presence of limonene by bromine test.

Chutia *et al*. (2009) suggested that the essential oil extracted from lemon peels is effective against several forms of pests like bacteria and fungi.

4.9.1.3 EXTRACTION OF BIO EXTRACT FROM PAPAYA PEELS

Figure 20 shows the Extraction of Bio extract from Papaya Peels.

FIGURE 20: EXTRACTION OF BIO EXTRACT FROM PAPAYA PEELS



Papaya peels **Bio extract from Papaya peels**

Figure 20 represents the bio extracted obtained from papaya peels using water as solvent. 1 part of bio extract from papaya peels was taken for the development of liquid laundry detergent.

Similar studies had been done by Aravind *et al.* (2013), Khan *et al.* (2012), and Kokila *et al.* (2016) also reported in their studies that these bioactive substances can be employed as antioxidants and antimicrobials, the majority of researchers are focused on finding an effective method to separate these bioactive substances from fruit peels.

Huet *et al.* (2006) reported that the peel of the papaya contains a variety of phytochemicals, particularly phenolic compounds that have antioxidant qualities. Chymopapain and papain, two significant physiologically active components in *Carica papaya,* are widely utilised to treat digestive issues.

Durán *et al.* (2005) and Kokila *et al.* (2016) stated that vitamins, amino acids, carbohydrates, carotene, lycopene, and polyphenols are abundant in papaya peel extracts. The riboflavin found in papaya peel extracts helps to generate the bound co-enzymes flavin mono nucleotide (FMN) and flavin adenine dinucleotide (FAD), which serve as catalysts for a variety of oxidation and reduction reactions.

4.9.1.4 PRODUCTION OF SOAP NUT LIQUID

FIGURE 21: PRODUCTION OF SOAP NUT LIQUID

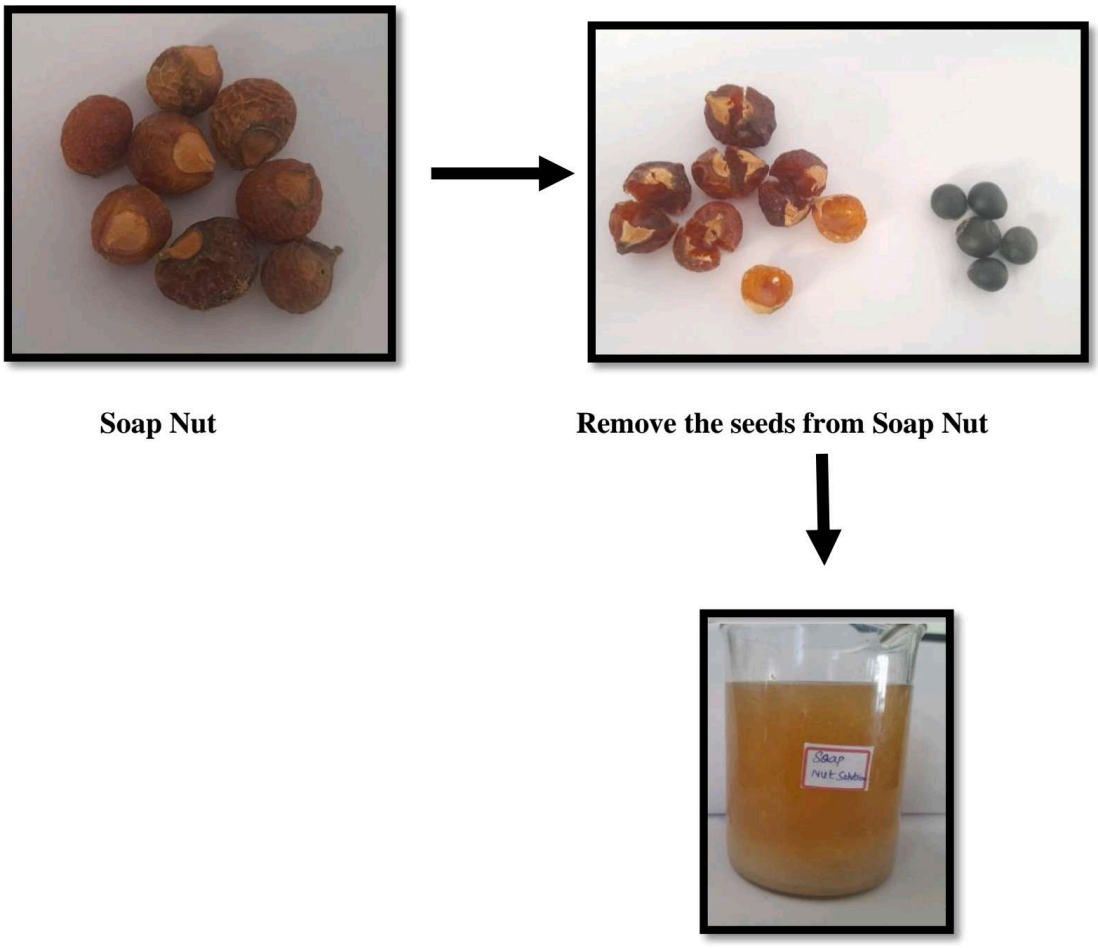


Figure 21 represents the production of soap nut liquid from soap nut. From the soap nut, seeds were removed and boiled for 20-30 minutes and filtered. 5 parts of the filtered soapnut liquid are taken for product development.

Velusamy *et al*. (2021) reported that soapnut seeds biochar was developed and employed as an adsorbent for the efficient removal of pollutants and newly emerging toxins, such as ciprofloxacin-500mg, from wastewaters produced by pharmaceutical enterprises.

Panda *et al*. (2020) suggested that due to their high concentration of hydrophilic and low concentration of hydrophobic groups, soapnuts function as natural surfactants. Because of its physical and chemical characteristics, soapnut seeds are a wetting agent. Due to the

high concentration of O-H groups on its surface, it demonstrates antioxidant and antibacterial characteristics.

4.9.1.5 EXTRACTION OF ESSENTIAL OIL FROM *OCIMUM BASILICUM*

FIGURE 22: EXTRACTION OF ESSENTIAL OIL FROM *OCIMUM BASILICUM*

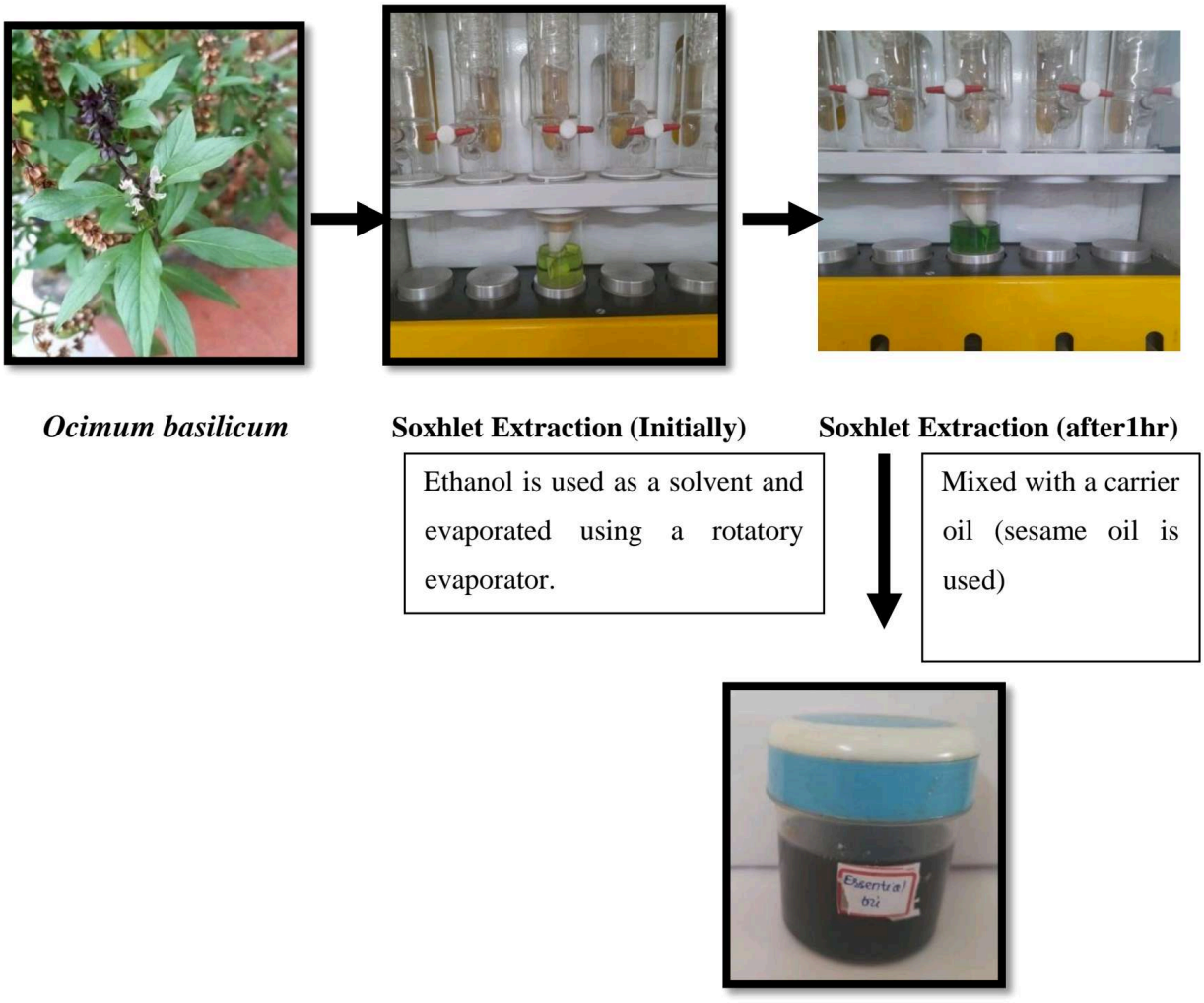


Figure 22 exhibits the process for obtaining the essential oil from *Ocimum basilicum* by the soxhlet extraction method. The extraction is performed till the completion of two cycles. 3 to 4 drops were added to the developed product for fragrance purposes.

According to Telci *et al*. (2006), its oil is traditionally produced from leaves and blossoms and is widely utilised in food processing systems as a flavouring agent and in the pharmaceutical sectors for therapeutic purposes. According to Kathirvel *et al.* (2012) and Elgndi *et al*. (2017), the composition of basil essential oil considerably varies depending on

the season, growing area, and stage of growth. Eucalyptol, linalool, eugenol, methyl-chavicol, geraniol, methyl cinnamate, cadinol, camphor, and carvacrol are the primary oxygenated and aromatic oxygenated monoterpenes found in *Ocimum basilicum* essential oils.

4.9.1.6 ECO FRIENDLY LIQUID LAUNDRY DETERGENT

FIGURE 23: ECO FRIENDLY LIQUID LAUNDRY DETERGENT

Figure 23 represents the eco friendly liquid laundry detergent that is used for further analysis and for application studies. 10 parts of the partially purified enzyme, 1 part of limonene from lemon peels, 1part of bio extract from papaya peels, 3 drops of essential oil from *Ocimum basilicum*, and 5 parts of soap nut solution were present in the liquid laundry detergent. To the developed product, 12 percent salts are added which, act as a natural preservative, and also, it increases the shelf life of the product. All the above components are added in their respective ratios, and further analysis and application studies were done using the liquid laundry detergent.

4.9.2 ANALYSIS OF LIQUID LAUNDRY DETERGENT

4.9.2.1 pH

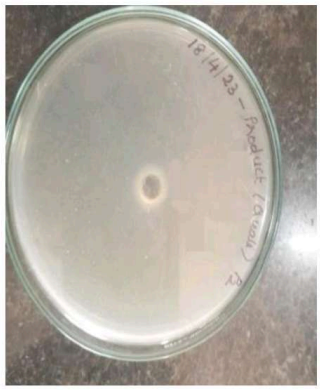
. pH 7.2 is obtained from the product, which shows it is slightly alkaline and also it is suitable for detergent purposes.

According to a study by Boonchai and Iamtharachai (2010), the pH of liquid cleansers, dishwashing liquids, a beauty bar, and alcohol gels ranged from acidic to neutral and were compatible with the normal skin pH. Most powdered laundry detergents bar soaps and baby soaps had pH values in the alkali range. Concentrated cleansers have a pH that is slightly different from their dissolved forms.

4.9.2.2 QUALITATIVE ANALYSIS (PROTEASE ASSAY)

Figure 24 represents the qualitative analysis of protease in liquid laundry detergent.

FIGURE 24: QUALITATIVE ANALYSIS (PROTEASE ASSAY)



From Figure 24, it was noted that a clear zone was observed around the wells with a diameter of 12 mm. This indicates that the liquid laundry detergent retains the protease activity.

According to AlGburi (2016), *B. subtilis* KATMIRA1933 cells or CFS showed proteolytic activity with clear zone areas of 13 ± 0.5/5 ± 0.3 mm after 24 hrs incubation.

4.9.2.3 FOAM TEST

Figure 25 represents the foam test for liquid laundry detergent.

FIGURE 25: FOAM TEST

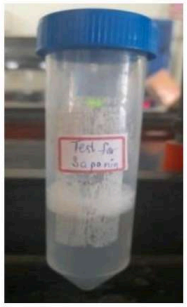


Figure 25 indicates stable foam forms, showing the presence of saponin. Because saponin is present in soap nut solution, the liquid laundry detergent has a foaming agent that helps with cleaning and also contains foaming agents.

Badi and Khan (2014) reported that small and dense foams were observed in herbal shampoo, which also contains soap nuts.

4.9.2.4 ANTIFUNGAL ACTIVITY

The antifungal activity present in the test samples is allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organism. *Candida albicans* is used as a test organism. Amphotericin B is used as a positive control. Wells were cut, and 10µl, 15 µl and 25 µl of the test samples were added. The diameter of the inhibition zone that formed around the well was measured after the plates had been incubated at 37°C for 24 hours to determine the antifungal activity, as shown in Table 9

TABLE 9

ZONE OF INHIBITION OF THE LIQUID LAUNDRY DETERGENT AGAINST *CANDIDA ALBICANS*

Samples (Volume)	**Zone of Inhibition (Units)**
Positive Control (50 µl)	1.6 ± 0.05
Test sample(10 µl)	0.6 ± 0.08
Test sample (20 µl)	0.8±0.04
Test sample (30 µl)	0.9 ± 0.02

From Table 9, it can be inferred from the results of the preliminary antifungal evaluation that liquid laundry detergent possesses good antifungal activity based on the zone of inhibition, and also it enhances the shelf life for a longer period of time.

Ongena *et al*. (2005) reported strongly and suggested that lipopeptides are responsible for most of the antifungal activity developed *in vitro* by *B. subtilis* toward multiple plant pathogens.

4.10 APPLICATION STUDIES

4.10.1 ACTION ON COAGULATED EGG WHITE

The partially purified proteases were tested for their ability to digest the albumin present in the egg white.

FIGURE 26: HYDROLYSIS OF NATURAL PROTEINS

The effect of an enzyme on coagulated egg white is depicted in Figure 26. Coagulated egg white that has not been treated with protease is visible in the first tube. The coagulated egg white was treated with the partially purified protease in the second and third tubes.

From Figure 26, it can be noted that the partially purified proteases from *Bacillus subtilis* were able to digest coagulated egg white, as shown in test 1 and test 2 and also we can confirm that the partially purified enzyme in liquid laundry detergent would possess the protease activity of the enzyme.

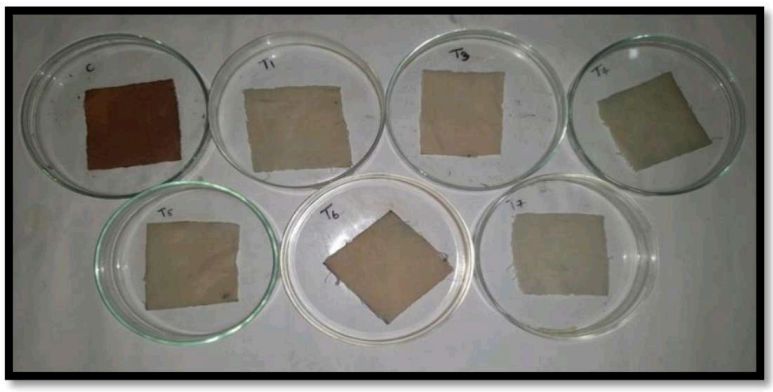
According to Kieliszek *et al*. (2021), proteolytic enzymes are utilised in the cheese-making process to coagulate milk proteins in addition to hydrolyzing proteins. Using proteolytic enzymes, several protein hydrolysates are produced from milk.

4.10.2 ACTION ON SOYA STAIN

Protease is a well-known detergent component that effectively eliminates stains from clothing. Many variables, including pH, temperature, and detergent composition, affect how well detergent proteases work.

FIGURE 27: DESTAINING OF SOYA-STAINED CLOTH AT VARYING TEMPERATURES

A) Destained soya-stained cloth at 37°C B) Destained soya-stained cloth at 40°C



C) Destained soya-stained cloth at 60°C

Figure 27 shows the destaining activity of soya stain using various samples at varying temperatures. Distilled water is used as a control, T1 is a commercial detergent, T2 is soya-stained cloth, T3 is detergent with partially purified enzyme, T4 is detergent with crude protease, T5 is detergent with liquid laundry detergent, T6 is only crude protease, T7 is only liquid laundry detergent are treated with soya stain at varying temperature.

When the stained cloth was washed with only distilled water, the stain was not removed. In T5 and T7, the stain was removed when treated with commercial detergent when compared with other test samples. This shows the efficacy of the liquid laundry detergent in destaining. When destaining was done at 40°C, maximum destaining occurred in T5 and T7 when compared to other temperatures.

Thus, it can be concluded from the facts above that the liquid washing detergent was able to completely remove the soya stain.

4.10.3 ACTION ON METHYLENE BLUE DYE

FIGURE 28: DESTAINING OF METHYLENE BLUE DYE AT VARYING TIME

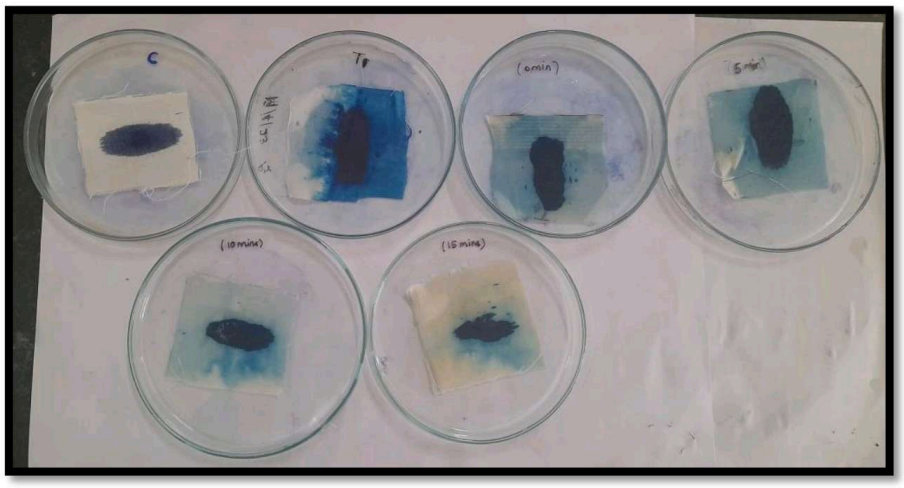


Figure 28 shows the methylene blue dye was destained using liquid laundry detergent at varying time periods (0min, 5mins, 10mins,15mins). To test the detergent's activity, the methylene blue dye-stained cloth was incubated at 37°C with liquid detergent at various time intervals. After 15 minutes of incubation, liquid laundry detergent was able to partially destain the colour. The ability of the liquid detergent to remove the staining pigment methylene blue was discovered to be only moderately, indicating that the liquid detergent had good detergent action.

According to Ramachandran and Arutselvi (2013), the partially purified *Nomuraea rileyi* protease had a high capacity for removing the staining colour methylene blue, indicating that the protease enzyme had significant detergent action.

Thus, it can be concluded from the facts above that the liquid washing detergent was able to moderately remove the methylene blue dye stain.

Summary & Conclusion

5. SUMMARY AND CONCLUSION

Protease is a type of enzyme that can be produced by all living things, as opposed to other enzymes, which only certain types of organisms can only produce. Proteolytic enzymes can be found in bacteria, animals, or plants (Singh *et al*., 2004). They are as proteolytic enzymes or proteinases (Rani *et al*., 2012).

Alkaline proteases are enzymes that function best when the pH is between 9 and 11. Protease is a type of enzyme that can be produced by all living things, as opposed to other enzymes, which only certain types of organisms can only produce (Sharma *et al.*, 2017). Many industries use alkaline protease, including the detergent industry, silver recovery from X-ray films, bleaching agents, hair removers in the leather industry, and protein degradation of prawn waste to make chitin (Nilegaonkar *et al*., 2007 and Shikha *et al*., 2007).

Bacillus subtilis showed higher proteolytic activity with a clear zone of 18 mm when gelatin than on casein or skimmed milk in nutrient agar medium. The zone of clearance for casein and skimmed milk as a substrate was 16mm and 11mm respectively. The maximum protease activity for the effect of carbon source was found to be galactose with protease activity (174.20 U/ml) followed by starch (67.69 U/ml), and sucrose showed least protease activity (41.072 U/ml).

In the present study, the maximum protease activity was found for the nitrogen source peptone, with protease activity (41.072 U/ml) For the effect of incubation time on protease production, maximum activity was noted at 48hrs of incubation (67.69 U/ml) followed by 72 hrs (41.07 U/ml). When incubated only for 24 hrs, the activity was significantly less and found to be (14.45 U/ml).

The ammonium sulphate precipitated samples were lower than the crude sample, with the 40-60% precipitated sample exhibiting the maximum activity (24.51 U/ml), followed by the 0-20% ammonium sulphate precipitated sample (14.48 U/ml). The specific activity of all the ammonium sulphate precipitated samples increased compared to crude (287.8 U/mg). The 60-80% ammonium sulphate precipitated sample registered the highest specific activity (946 U/mg), followed by the 40- 60% precipitated sample (817

U/mg). Regarding recovery percentage, the 40-60% ammonium sulphate precipitated sample gave the highest value (70.96%), followed by the 0- 20% (41.92%) ammonium sulphate precipitated sample.

The purification fold of the samples also followed a similar trend, with the 60-80% ammonium sulphate precipitated sample indicating the highest value (3.28), followed by the 40-60% ammonium sulphate precipitated sample (2.83). The 60-80% ammonium sulphate precipitated protease sample from *Bacillus subtilis* exhibited the best purification profile by means of the highest specific activity and purification fold.

The protein content of 60-80% ammonium sulphate precipitated protease was remained constant after dialysis. In contrast, the ammonium sulphate precipitated protease activity was found to be increased, with the highest value for ammonium sulphate precipitated protease (11.26 U/ml).

Regarding, specific activity, the value increased for the sample after dialysis with 60-80% ammonium sulphate precipitated protease exhibiting the maximum value 1126 U/mg. The recovery percentage of the ammonium sulphate precipitated protease sample increased (32.59%). On dialysis, the purification fold for the ammonium sulphate precipitated protease sample has increased (3.91) by confirming the purity of the isolated protease.

From the effect of pH for partially purified protease, it is seen that 100% relative activity was found at pH 9.0 with enzyme activity 3.62 U/ml, followed by 76% activity found at pH 8.0 with 2.76 U/ml enzyme activity and 52% activity at pH 10.0 with enzyme activity 1.89 U/ml. Thus the partially purified proteases have an optimum pH of 9.0 since the relative activity was 100% at that pH**.** For pH stability, optimum pH 9 showed the maximum value of protease 0.94 U/ml for 20 minutes of incubation followed by 60 mins of incubation, 0.89 U/ml. It can be inferred that partially purified protease has an optimum pH of 9 and is stable for 20mins.

From the effect of temperature for partially purified protease, it is seen that 100 % relative activity was found at 40°C with enzyme activity 4.42 U/ml, followed by 90% relative activity at 50°C and 60°C with enzyme activity 3.98 U/ml. Thus the partially purified proteases have an optimum temperature of 40°C since the relative activity was 100% at that temperature. For the stability of temperature, the optimum temperature showed the maximum value of protease 1.58 U/ml at 45 minutes of incubation, followed by 60 mins of incubation

with enzyme activity 1.47 U/ml. It can be inferred that partially purified protease has an optimum temperature of 40°C and is stable for 45 mins.

The components present in the products are partially purified enzyme from *Bacillus subtilis,* limonene from lemon peels, bio extract of papaya peels, soap nut solution, and essential oil from *Ocimum basilicum* (10:1:1:5). 12 percent salt is added, which acts as a natural preservative and increases the product's shelf life. Bromine test confirmed the presence of limonene.

Eco friendly liquid laundry detergent shows pH 7.2, indicating a slightly alkaline nature and, the foam test confirms the presences of saponin. From the qualitative analysis, we can conclude that protease activity remains in the liquid laundry detergent. It also possesses antifungal properties based on the results of the zone of inhibition.

From application studies, the partially purified proteases from *Bacillus subtilis* could to digest coagulated eggs. Destaining activity was used in soya stained cloth where the stain was not able to remove when treated with distilled water. In stained clothes T5 (detergent with liquid laundry detergent) and T7 (only liquid laundry detergent) removed stains like treated with commercial detergent while comparing with other test samples. This shows the efficacy of the liquid laundry detergent in destaining. When destaining was done at 40°C, maximum destaining occurred in T5 and T7 compared to other temperatures.

To test the detergent's activity, the methylene blue dye- stained cloth was incubated at 37°C with liquid detergent at various intervals. After 15 minutes of incubation, liquid laundry detergent could to partially destain the colour. The ability of the liquid detergent to remove the staining pigment methylene blue was discovered to be only moderate, indicating that the liquid detergent had good detergent action.

Future Recommendations

1. Purified dialysed protease can be used in laundry detergent to replace partially purified protease, and its effect can be observed.
2. Various application studies can be done using different stains.
3. Ratio of components can be increased to check the efficiency of the product.

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Appendices

APPENDIX I

PREPARATION OF INOCULUMS

(Pant *et al*., 2015)

Materials/ Reagents

1. 50 ml of Nutrient Broth (g/L)
2. *Bacillus subtilis* – bacterial strain
3. Nutrient Agar
4. Medium (pH 8) – 1% glucose, 0.5% casein, 0.55% yeast extract, 0.2% KH_2PO_4, 1% Na_2CO_3 , 0.2% $MgSO_4.7H_2O$
5. Inoculation Loop
6. Spirit Lamp

Procedure

1. A loopful culture of *Bacillus subtilis* strain was inoculated into a 50ml nutrient broth (g/L)
2. The Culture was incubated for 72 h at 37°C under shaking condition (150rpm).
3. 1 ml of this broth culture was used as the inoculums.
4. Colonies were sub cultured and maintained to obtain a pure culture on nutrient agar plates, which was transferred to a medium (pH 8.0) containing 1% glucose, 0.5% casein, 0.55% yeast extract, 0.2% KH_2PO_4, 1% Na_2CO_3, 0.2% $MgSO_4 \cdot 7H_2O$, pH 8 and incubated at 37 °C with continuous shaking.

APPENDIX II

PREPARATION OF SKIM MILK AGAR

(Pailin *et al*., 2001, Mohanasrinvasan *et al*., 2012)
[Modified Procedure]

Materials/ Reagents

1.1g of non fat dry milk

2. Distilled water

3. Nutrient Agar

Procedure:

1.Skim milk agar medium is used for primary screening of protease by mixing 1 g of non fat dry milk was mixed with 50 ml of distilled water.

2. The mixture was stirred thoroughly and autoclaved at 121°C for 15 min.

3. The pH of the medium adjusted by using the pH meter maintained at pH 6, the suspension of 2.5% of agar (1.25g of agar in 50ml of skim milk) and used for solidification were autoclaved at 121°C for 15 min.

4. For plating, skim milk agar solutions were held in a water bath at 50°C and mixed thoroughly.

5. The skim milk agar was poured quickly into plates then kept at 4°C until used.

[Modifications – instead of 25 g of non fat dry milk in 250 ml of distilled water, 1g of non fat dry milk in 50 ml of distilled water is used]

APPENDIX III

SUBSTRATE SCREENING

(Pant *et al*., 2015)

Materials/ Reagents:

1. Gelatin
2. Casein

3. Skim Milk Agar
4. Nutrient Agar
5. Pure Culture Isolates - *Bacillus subtilis*

Procedure:

Method I

1. Three different substrates (Gelatin, Casein ,Skim Milk) at 1% (w/v) was then added to nutrient agar medium, sterilized and poured into Petri plates.

2. Wells were made with a sterile cork borer, and 100–200µL of cell-free culture filtrate were dispensed aseptically into each well and incubated at 37 °C.

3. After 12 h, enzyme activity was visualized as clear zones around the wells due to hydrolysis of substrates in the presence of indicator solution, and the diameter of the proteolytic zone was measured

Method II

1. Gelatin and Casein were used as the substrate in nutrient agar medium.

2. The culture was streaked in a straight line and incubated at 37 °C.

3. After 12 h, the plates were flooded with suitable indicators

Indicator for Gelatin: 15% $HgCl_2$ solution in 20% HCl for 5 min

Indicator for Casein: saturated ammonium sulfate is used for 5 min.

APPENDIX IV

SHAKE FLASK FERMENTATION WITH SELECTED MEDIUM

(Pant *et al*., 2015)

Materials/ Reagents:

1. Optimized Production Medium (pH 8.0):1% galactose, 0.5%casein, 0.55% peptone, 0.2% KH_2PO_4 , 1%Na_2CO_3 , 0.2% $MgSO_4.7H_2O$

2. 4% inoculums - *Bacillus subtilis*

Procedure:

1. Alkaline protease was produced in an optimized production medium of pH 8.0 containing 1% galactose, 0.5% casein, 0.55% peptone, 0.2% KH_2PO_4, 1% Na_2CO_3 and 0.2% $MgSO_4 \cdot 7H_2O$.

2. 4% inoculums was added to the production medium and incubated with continuous shaking.

3. Shaker fermentations were carried out at 37 °C for 72 h with controlled agitation at 100–150 rpm.

4. At the end of the fermentation period, the whole culture broth was centrifuged at 5000 rpm for 30 min to remove debris, and the supernatant was collected and used for further experiments.

APPENDIX V

PRECIPITATION OF PROTEINS BY AMMONIUM SULPHATE FRACTIONATION

(Simpson, 2006)

Principle

Ammonium sulphate is a particularly useful salt for the fractional precipitation of proteins. It is available in a highly purified form, has great solubility allowing for significant changes in the ionic strength and is inexpensive. Changes in the ammonium sulphate concentration of a solution can be brought about either by adding solid substance or by adding a solution of known saturation, generally a fully saturated (100%) solution.

Materials/Reagents

1. Ammonium sulphate – Add ammonium sulphate to the protein solution as a solid.
2. Resuspension buffer – Resuspend the concentrated protein with 20 mM Tris HCl after ammonium sulphate precipitation.
3. Protein sample – Crude extract.

Procedure

1. Use a graduated cylinder to measure the volume of the protein solution to be concentrated. Pour the solution into a beaker with a capacity approximately twice the measured volume of the protein solution. Place the beaker in ice.

2. Weigh the necessary amount of ammonium sulphate required to give the desired saturation.
3. Place the beaker containing the protein solution (and a large magnetic stir bar) on a large stir plate in the cold room.
4. Slowly add the solid ammonium sulphate to the stirred protein solution and allow the mixture to stand for 60 minutes to ensure complete precipitation.
5. Transfer the mixture to screw-cap polycarbonate centrifuge tubes and carefully balance them. Ensure that the centrifuge tubes are balanced within 0.1-0.2 g across the rotar axis.
6. Centrifuge the tubes at 10,000 g for 15 minutes at 4^{o}C.
7. Decant the supernatant solution, saving both the protein pellet (0 -20%) and the supernatant.
8. Return the supernatant to the cold room and slowly add ammonium sulphate to the supernatant to achieve a concentration of 40%. Stir the mixture for 60 minutes to ensure complete precipitation.
9. Transfer the solution to the appropriate centrifuge tubes and centrifuge the solution at 10,000 g for 15 minutes at 4^{o}C.
10. Decant the supernatant solution, saving both the protein pellet (20- 40% fraction) and the supernatant.
11. Repeat steps 8-10, adding ammonium sulphate to concentrations of 60%, 80%, and so on, up to 100% saturation, removing precipitated proteins by centrifugation at each step before increasing the ammonium sulphate concentration.
12. Resuspend the recovered protein pellets (0-20, 20-40, 40-60, 60-80 and 80-100 %) in a resuspension buffer (20 mM TrisHCl buffer, pH 7.8) and assay them for total proteins.

S.No	Final concentration (%)	Ammonium sulphate (g/ 100 ml aliquots)
1	0-20	5.35
2	20-40	11.45
3	40-60	36.60
4	60-80	52.30
5	80-100	70.70

APPENDIX VI

DESALTING OF PROTEINS BY DIALYSIS

(Roe, 2001)

Principle

Dialysis is commonly used for removing salts from proteins. The presence of salts in proteins interferes in many ways. Special semi permeable membranes called dialysis tubes have the property to allow compounds with small molecular weights to pass through them, while those with high molecular weights like protein are held back.

Materials/Reagents

1. Dialysis bags (mm)
2. Magnetic stirrer and stirrer motor
3. 2% Sodium bicarbonate
4. 0.05% EDTA
5. 20% Ethanol
6. 0.1% Sodium azide
7. Distilled water
8. 20 mM TrisHCl buffer (pH 7.8)

Procedure

1. Wash hands with distilled water and ethanol before handling the dialysis bags.

2. Select dialysis tubing of suitable diameter and pore size cut into suitable lengths to contain the volume required.

3. Boil the dialysis bags in 100 ml of 2 % Sodium bicarbonate and 0.05 % EDTA for 15 minutes.

4. Discard the solution and boil for 10 minutes in distilled water.

5. Wash again in distilled water and boil for 20-60 minutes in distilled water without touching the sides of the beaker.

6. Prior to use, rinse the dialysis tubing inside and outside with distilled water and buffer.

7. Seal one end of the tubing with a double knot or dialysis clip. Check for leakage using distilled water.

8. Each protease sample to be desalted is taken inside the dialysis bag and the other end of the bag is secured tightly with a knot of thread or dialysis clip to prevent leakage. The bags should be half full only
.
9. Suspend the bags in a large beaker containing 20 mM TrisHCl buffer (pH 7.8) and keep stirring the contents at 4°C with the help of a magnetic stirrer.

10. Salt molecules pass freely and get diluted by the large volume of fluid in the external medium.

11. Repeat changes of the buffer every 8 hours.

12. The samples were taken using syringe and used for further experiments

13. Wash the used bags using 0.1 % sodium azide for reuse.

APPENDIX VII

ESTIMATION OF PROTEIN

(Lowry *et al*., 1951)

Protein can be estimated by different methods as described by Lowry and also by estimating the total nitrogen content. No method is 100% sensitive. Hydrolysing the protein and estimating the amino acids will give the exact quantification. The method developed by (Lowry *et al.,*1951) is sensitive enough to give a moderately constant value and hence largely followed. Protein content of enzyme extracts is usually determined by this method.

Principle

The blue colour developed by the reduction of the phosphomolydic phosphotungstic components in the Folin – Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reduction of the protein with the alkaline cupric tartarate are measured in the Lowry's method.

Materials/Reagents

1**. Reagent A:** 1% Copper sulphate

2 **.Reagent B:** 2% Sodium Potassium Tartarate.

3. **Reagent C:** 2% sodium carbonate in 0.1 N sodium hydroxide.

4. **Reagent D:** Mixed just before use,1ml of Reagent A and,1ml of Reagent B and 100ml of Reagent C

5. **Reagent E:** Folin – Ciocalteau reagent (Mixed equal volumes of commercially available reagent and distilled water just prior to use).Stored and protected from light.

6.**Protein Solution** (Stock Standard) :Weigh accurately 50 mg of bovine serum albumin (Fraction V) and dissolve in distilled water and make up to 50 ml in a standard flask.1ml of this solution contains 1mg of protein.

7. **Working Standard**

Dilute the stock solution in the ratio 1:10 for use as working standard.

One ml of this solution contains 100 μg protein.

Procedure

1. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard into a series of test tubes.
2. Pipette out 1.0 ml of the sample extract in two other test tubes.
3. Make up the volume to 1 ml in all the test tubes. A tube with 1 ml of water serves as the blank.
4. Add 3 ml of reagent C to each tube including the blank. Mix well and allow to stand for 10 minutes.
5. Then add 0.3 ml of reagent D, mix well and incubate at room temperature the dark for 30 minutes. Blue colour is developed

6. Take the readings at 660 nm.
7. Draw a standard graph and calculate the amount of protein in the sample

APPENDIX VIII

ENZYMATIC ASSAY OF PROTEASE

(Anson, 1939; Folin and Ciocalteau, 1927).

Principle

Protease hydrolyses casein in the presence of water to give amino acid.

Casein + H_2O —------>Amino Acids

Materials/Reagents

1. **Reagent A:** 20 mM TrisHCl buffer, pH 7.8 at 37°C.
2. **Reagent B:** 0.65% Casein solution

   0.65 g casein dissolved in 100 ml of Reagent A. Adjust the pH to 7.8.

3. **Reagent C:** 110 mM Trichloroacetic acid reagent (TCA)

   1.8 g of TCA dissolved in 100 ml of deionized water.

4. **Reagent D:** Folin Ciocalteu's Phenol Reagent

Dilute 10 ml Folin Ciocalteau's Phenol Reagent to 40 ml with deionized water.

5. **Reagent E:** 500mM Sodium Carbonate Solution.

   5.3 g of sodium carbonate dissolved in 100 ml of deionized water.

6. **Reagent F:** Enzyme solutions: crude extract, ammonium sulphate precipitated,dialyzed.

7. **Reagent G:** 1 mM L- Tyrosine Standard

Dissolve 0.0199 g of L-Tyrosine in 100 ml of deionized water and heat gently (do not boil) until the tyrosine dissolves and cool to room temperature.

Procedure

Pipette the following reagents into suitable vials (in milliliters)

S.No	Reagents	Test (ml)	Blank (ml)
1	Reagent B (Casein)	5.0	5.0
Equilibrate to 37 °C.			
2	Reagent F (enzyme solution)	1.0	-
Mix by swirling and incubate at 37 °C for about 10 minutes			
3	Reagent C (TCA)	5.0	5.0
4	Reagent F(Enzyme solution)	-	1.0
Mix by swirling and incubate at 37 °C for about 30 minutes			
5	Filter through Whatman filter paper No. 50 or 0.45 µm and use the filtrate for colour development		

Colour development

Prepare a standard curve by pipetting the following reagents into suitable vials (in milliliters).

S.No	Reagents	Standard 1	Standard 2	Standard 3	Standard 4	Standard blank 5
1	Reagent G (standard solution)	0.05	0.10	0.20	0.40	0.00
2	Deionized water	1.95	1.90	1.80	1.60	2.00
3	Reagent E (sodium carbonate)	5.00	5.00	5.00	5.00	5.00
4	Reagent D(F-C)	1.00	1.00	1.00	1.00	1.00

Sample

1. Pipette the following reagents into 4 vials (in milliliters):

S. No	Reagents	Test (ml)	Blank (ml)
1	Test filtrate	2.00	-
2	Blank filtrate	-	2.00
3	Reagent E (Sodium carbonate)	5.00	5.00
4	Reagent D (F-C)	1.00	1.00

2. Mix by swirling and incubate at 37°C for 30 minutes.
3. Remove the vials and allow them to cool to room temperature.

Filter through a 0.45 µm filter immediately prior to reading. Read the absorbance at 660 nm for each of the vials in suitable cuvettes.

Calculation

Standard curve:

δ A660 nm Standard = A660 nm standard – A660 nm standard blank .

Plot the standard δA660 nm Standard vs µ moles of tyrosine

Sample Determination:

δ A660 nm Sample = A 660 nm Test – A 660 nm Sample Blank

Determine the µ moles of tyrosine equivalents liberated using the standard curve.

$$\text{Units / ml enzyme} = \frac{(\mu \text{ Mole tyrosine equivalents released}) \times (\text{Total volume in millilitres of assay})}{(\text{Time of assay in minutes as per the unit definition}) \times (\text{Volume of enzyme in milliliters}) \times (\text{Volume used in colorimetric determination})}$$

$$\text{Units/mg solid} = \frac{\text{Units/ml enzyme}}{\text{mg solid / ml enzyme}}$$

$$\text{Specific Activity (or) Units / mg protein} = \frac{\text{Units/ml enzyme}}{\text{mg protein / ml enzyme}}$$

Unit definition:

One unit will hydrolyze casein to produce color equivalent to 1.0 µ mole (181µg) of tyrosine per minute at pH 7.5 at 37°C.

Final Assay Concentration:

In a 6 ml reaction mix, the final concentrations are 42 mM potassium phosphate, 0.54% (w/v) casein, 1.7 mM sodium acetate, 0.8 mM calcium acetate and 0.1 – 0.2 unit protease

APPENDIX IX

DETERMINATION OF OPTIMUM pH FOR PROTEASE

(Wang *et al.,* 2010)

Materials/Reagents

1. Samples – ammonium sulphate precipitated protease
2. Buffers at different pH

Prepare buffers of varying (pH 6.0 - 10.0).

pH	Buffers
6.0	50 mM Phosphate citrate
7.0	50 mM Phosphate
8.0-9.0	50 mM Tris-HCl
10.0	50 mM Glycine- NaOH

Procedure

1. Assay the protease activity with the samples at a temperature of 60˚C for 20 minutes as given in Appendix VIII.

2. Calculate the remaining activity / relative activity in percentage with reference to the activity of the optimum pH (consider the optimum pH activity as 100%).

APPENDIX X

PREPARATION OF BUFFERS

(Rao and Raghuramulu,1998)

1. Citrate Phosphate Buffer

Stock solutions: A: 0.1 M solution of citric acid (19.21 g/l).

B: 0.2 M solution of dibasic sodium phosphate (53.65g of $Na_2HPO_4.7H_2O$ or 71.7 g/l of $Na_2HPO_4.12H_2O$)

X ml of A + Y ml of B is diluted to a total of 100 ml.

X	Y	pH
17.9	32.1	6.0
6.5	43.5	7.0

2. Tris-HCl Buffer

Stock solution: A: 0.2 M solution of Tris - (hydroxymethyl) aminomenthane (24.2 g/l).

B: 0.2 M HCl.

50 ml of A + Y ml of B is diluted to total of 100 ml.

Y	pH
26.8	8.0
5.0	9.0

3. Glycine – NaOH Buffer

25 ml of 0.2 M Glycine (15.015 g / l) + Y ml 0.2 M NaOH is diluted to 100 ml

Y	pH
16.0	10

APPENDIX XI

DETERMINATION OF OPTIMUM TEMPERATURE FOR PROTEASE

(Wang *et al.,* 2010)

[Modified procedure]

Materials/Reagents

1. Samples – ammonium sulphate precipitated protease

Procedure

1. Assay the protease activity with the samples at varying temperatures (20, 30, 40, 50, and 60˚C) for 20 minutes at pH 9.0 using 20 mM Tris HCl buffer according to the procedure given in Appendix VIII.

2. Calculate the remaining activity / relative activity in percentage with reference to the activity of the optimum temperature (consider the optimum temperature activity as 100%).

APPENDIX XII

EXTRACTION OF LIMONENE FROM LEMON PEELS

(Davidowski and DiMarco, 2009)

[Modified Procedure]

Materials and Reagents

1. Razor Blade
2. Methanol
3. Lemon

Procedure

1. Samples of lemon rinds were carefully collected using razor blade.

2. The samples were checked to ensure that none of the white flesh under the rind was included in the sample.

3. The white flesh contributes to the mass of the sample but contains little limonene, this makes the rinds appear to have a lower limonene concentration.

4. Then each sample was cut down to a mass of approximately 0.1 g.

5. The rind samples were each placed in 7 ml vials with 5ml methanol. The vials were shaken vigorously for 5minutes and then allowed to stand for an additional 5 minutes.

6. After the 10 minutes extraction was complete, 0.5 ml aliquots of methanol from each vials were diluted volumetrically (20:1 for lemon).

7. The diluted samples were used for the experiments

APPENDIX XIII

EXTRACTION OF BIOEXTRACT OF PAPAYA PEELS

(Balavijalakshmi and Ramalakshmi, 2017)

Materials and Reagents

1. Papaya peels

2. Distilled Water

Procedure

1. The papaya peels are washed thoroughly with distilled water to remove visible dust particles and air dried at room temperature to remove the water molecules.

2. About 25g of dried and finely sliced peels are taken and added into 100ml of distilled water and heated for 30min.

3. The crude extract is then filtered out using Whatman No. 1 filter paper to get a clear bio extract.

APPENDIX XIV

PRODUCTION OF SOAP NUT LIQUID

(Fran,2023)

Materials and Reagents

1. Soap Nuts
2. Distilled Water

Procedure

1. Place 4 – 6 crushed deseeded soapnuts with 2 cups of water in a beaker.

2. Bring to boil, turn down the flame and let it simmer for 20 -30 minutes.

3. Strain the liquid, put the soapnuts to dry (they can be reused to make more soap liquid) and once cold pour the soapnut concentrate in a bottle..

APPENDIX XV

TESTING OF PROTEASE WITH COAGUALATED EGG WHITE

(Najafi *et al*., 2005)

Principle

Protease can hydrolyze the protein present in coagulated egg white.

Materials/Reagent

1) 20 mM TrisHCl (pH 9.0)
2) Coagulated egg white
3) Partially purified protease

Procedure

1) Take 3 pieces of coagulated egg white in three separate test tubes.

   Label 2 as ‘Tests’ and one as ‘Control’.

2) Add 3 ml of the buffer to the ‘Control’ tube and 2 ml to the ‘Test’tube.

3) Add 1 ml of the partially purified protease to each of the 'Test' tubes.

4) Incubate the tubes at 60˚C overnight

5) Note the changes after incubation.

APPENDIX XVI

TESTING OF PROTEASE FOR DESTAINING

(Najafi*et al*., 2005)

[Modified Procedure]

Principle

Protease has the ability to destain soya stain cloth.

Materials/Reagents

1. Distilled water
2. Detergent powder
3. Soya sauce
4. Cotton cloth pieces
5. Partially Purified protease enzyme
6. Crude Protease
7. Developed product

Procedure

1. Stain seven clean pieces of white cloth (5x5 cm) with soya sauce and prepare the following sets to study the destaining property of protease.
    i. Soya sauce stained cloth dipped in flask with distilled water (100 ml)
    ii. Soya sauce stained cloth dipped in flask with distilled water (100 ml)+ 1 ml detergent (7mg ml^{-1})
    iii. Soya sauce stained cloth dipped in flask
    iv. Soya sauce stained cloth dipped in flask with distilled water (100 ml)+ 1 ml partially purified enzyme (7mg ml^{-1})

v. Soya sauce stained cloth dipped in flask with distilled water (100 ml) + 1 ml detergent (7mg ml^{-1}) + 2 ml developed product.

vi. Soya sauce stained cloth dipped in flask with distilled water (100 ml) + 2 ml crude enzyme protease with developed product expect partially purified.

vii. Soya sauce stained cloth dipped in flask with distilled water (100 ml) + 2 ml crude developed product.

2. Incubate all the seven flasks at 60˚C for 15 minutes.
3. After incubation, rinse cloth pieces with water and then dry.
4. Observe the cloth for destaining.