CHAPTER 1

INTRODUCTION

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1.1. Freshwater mud eel (*Monopterus cuchia*)

The freshwater mud eel *Monopterus cuchia* is an amphibious fish also known as rice eel, gangetic mud eel, or swamp eel belonging to Synbranchidae [1]. It is a freshwater air-breathing fish commonly found in freshwater and brackish water with an altitudinal range of 76-1350 m above sea level [2, 3]. *M. cuchia* is naturally distributed across North-East and Northern India, Sri Lanka, Pakistan, Bangladesh, Philippines, Nepal, Indonesia, and New Guinea [4, 5]. The fish is pollution resistant and shows adaptations to adverse environmental conditions such as low O_2 content, shallow water, and temperature elevations [6]. It is a tasty, nutritionally rich, economically and medicinally important freshwater fish that could play a vital role in the growth of the human body [7]. *M. cuchia* plays an important role in the socioeconomic development of developing countries by generating employment and boosting the economy. It has been reported that a large section of people is involved in its production, marketing, and other associated business [8]. Tribal communities *viz*., Hajong, Garo, Manipuri, Shaotal, and Rajbongshi of North-East India traditionally use *M. cuchia* for the treatment of various ailments due to their high therapeutic value [9]. Owing to its high nutritional and medicinal values *M. cuchia* has a great demand in foreign countries and thus, plays a significant role in export earnings and international trade [10].

1.2. Habitat

The fish is commonly available in the muddy banks of water bodies including ponds, canals, rice fields, swampy areas, etc. [2]. It also inhabits ditches and temporary pools and can survive there for about 4-5 months during summer [11]. The fish is nocturnal in habit, they spend the day hiding behind the rocks and mud [12]. They survive the entire summer living in the mud holes. The respiratory organs help in gaseous exchange in water-deficient muddy areas [12].

1.3. Morphology

Cuchia has a cylindrically elongated body. The maximum length and weight of the fish were reported to be 62.7 cm and 547.0 g, respectively [3]. The skin of the fish is very thick and slippery. The dorsal and ventral parts of the body are brownish and yellowish

brown, respectively. Numerous black spots are present all over the body. The head of the fish is triangular and is covered with numerous spots. More than 22-28 lines are present at the ventral part of the head. The fish has small eyes which are covered by translucent layer of the skin [3]. The females of *M. cuchia* are larger than the males [13]. The slippery nature of the fish due to the high amount of mucus enables it to suck its prey coming in close contact with the surface of respiratory epithelium [2]. The anus is situated posteriorly than the normal position. Only gill opening is situated at the anterolateral part of the body.

1.4. Systematic position

The classification of *M. cuchia* is shown in Table 1.1

Table 1.1 Classification of *M. cuchia*

Scientific classification of *M. cuchia*	
Kingdom	Animalia
Phylum	Chordata
Sub-Phylum	Vertebrata
Class	Oteichthyes
Infra-Class	Actinopterygii
Order	Synbranchiformes
Family	Synbranchidae
Genus	*Monopterus*
Species	*M. cuchia*
Synonyms	*Ophichthys cuchia, Amphipnous cuchia, Pneumabranchusalbinus, Pneumabranchusleprosus, Pneumabranchusstriatus, Unibranchapertura cuchia*

1.5. Reproductive cycle

The developmental stages in the testis of *M. cuchia* are spermatogonia, spermatocytes, spermatids and spermatozoa. Whereas, ovary have three developmental stages such as, oogenesis, and vitellogenesis stage as described below:

1.5.1. Male reproductive cycle

1.5.1.1. Spermatogonia

During the spermatogenesis process these are the first cells group to appear and hence densely found near the germinative zone of the mature male gonad. Morphologically these are spherical containing chromatin material and nucleoli with distinct nuclear wall. These cells form primary spermatocytes by undergoing mitotic division [14].

1.5.1.2. Spermatocytes

The first maturation division of the spermatogonia produces secondary spermatocytes. Both the primary and secondary spermatocytes are similar in size with an average diameter of 8.4 µm. The secondary spermatocytes then undergo second maturation division to give rise to spermatid [3].

1.5.1.3. Spermatids

The spermatids were tiny rounded bodies with a diameter of 5.3 µm. They have a less cytoplasm and most of their body was occupied by nucleus. These haploid, immature male cells are formed as a result of the meiosis (second division) of the spermatocytes.

1.5.1.4. Spermatozoa

The spermatids finally undergo morphological changes to give rise to spermatozoa. These are almost spherical containing a short tail. This stage becomes prominent from the month of May to June.

1.5.2. Female reproductive cycle

1.5.2.1. Oogenesis

Oogenesis take place at the outermost surface of the ovaries. The germ cells called oogonium undergo repeated mitotic divisions to increase in number. The mitotic divisions

then finally form the primary oocyte and ova. The developing ova is radially distributed with the immature one at the germinal zone and mature one at the periphery [15].

1.5.2.2. Oogonia

In this developing stage, gonad was attached to the dorsal peritoneum and the development of the basophilic oocytes occurs. These cells are small and round in shape containing a single distinct nucleolus. Oogonia mature into previtellogenic oocytes [14].

1.5.2.3. Previtellogenic oocyte

Depending on the size the previtellogenic oocyte are divided into two groups:

1.5.2.3.1. Early perinucleolus stage

The stage is associated with the oocyte growth and the size of the nucleus increases. The cytoplasm becomes homogenous and dense. In this stage the follicular layer is not noticeable. However, in the month of January perinucleolus was observed [3].

1.5.2.3.2. Late perinucleolus stage

In this stage the oocyte was enlarged and the cytoplasm is basophilic and homogenous. Nucleus is clearly visible and the follicular cells begins to develop throughout the oocyte. This stage was observed from January to mid-February [15].

1.5.2.4. Vitellogenesis

In this stage the synthesis of yolk occurs in the oocyte and substantial changes are observed in the nucleus, ooplasm and nucleolus. This stage was observed from the month of April to May.

The maximum mean gonad weights in females and males are recorded in May. In addition to this, the development of mature testis and oocytes was prominent from May to July and this species breeds only once a year [15]. In the case of males, the testis is soft, elongated, and dorsally attached to the body cavity. Testis consists of several seminiferous tubules where spermatogenesis takes place. It opens through the urogenital aperture via the spermatic duct which is formed by the union of posterior-ventrally located as vasa deferens. The mature testis of *M. cuchia* is creamy white while the ovary is single-lobed

and cylindrical. A thin mesentery connects the elongated and cylindrical ovary lobe along their dorsal surfaces. The mature ovary is yellowish whereas the immature one is compact and cream colour [15].

1.6. Food habit and the enzyme present in *M. cuchia*

The fish is nocturnal and carnivorous in feeding habit, therefore, prefers animal-based food like small fish, larvae, echinoderms, invertebrates, earthworms, snails, tubifex, fish fingerlings, amphibians, crustaceans, insect pupae, slaughterhouse waste, etc. [6]. The proteinaceous food is digested by the presence of endogenous enzymes produced in the digestive tract of the fish.

1.7. Therapeutic value

The fish is a major component of the diet of anaemic and weak people. Traditional healers believe that consumption of this species increases haemoglobin content of blood and enhances physical strength in ailing persons [16]. The fish is nutritionally rich with high medicinal value and has also been reported to be an important ingredient in traditional therapeutics of the North-Eastern states of India by different tribes and communities [17]. Studies on the plasma composition of the fish established the presence of 3.3-3.7 g, 67.3-72.5 mg, and 224.8-257.0 mg of protein, glucose, and triglycerides per 100 ml of blood, respectively [15]. The use of cuchia blood in combination with ethnomedicinal plants for the treatment of diabetes, asthma, anaemia, piles, and weakness have also been reported [18]. Moreover, Naga tribes from Nagaland has been reported to drink the fresh blood of the fish to cure various severe ailments [19]. The dried head of freshwater mud-eel is cooked with *Garcinia pedunculata* and subsequently consumed to treat haemorrhoids [20].

1.8. Economic importance

The fishery sector plays a significant role in an economic system by increasing living standards, influencing socioeconomic development, providing a livelihood for economically backward people, and generating employment [21]. Export earnings of cuchia is vital in markets [3]. The majority of the poor people find employment in the collection, production, transport, and marketing of the freshwater mud eel [1]. Accordingly, employment and income-generating opportunities can be developed in both

urban and rural areas [1]. *M. cuchia* is in great demand in the international markets due to its therapeutic and nutritional value [1].

1.9. Application of enzyme in industries: A green approach

Enzymes are biological catalyst that attract researchers for their huge catalytic power and green nature. Certain characteristics of enzymes *viz.*, nominal by-product formation, moderate reaction conditions, and substrate specificity makes them the most important entities for bioprocessing industry [22]. Widespread use of noxious chemicals in various industries have affected the human health as well as the environment [23]. Traditional leather processing generates huge quantity of liquid wastes, solid wastes (lime, and chrome dirt) and emits toxic gases such as volatile organic compounds, ammonia, hydrogen sulfide, etc. causing immense environmental problems [24, 25]. Furthermore, conventional detergents are mainly composed of xenobiotic compounds and hazardous chemicals such as bleaching agents, whitening agents, bluing agents, perfumers, anti-re-deposition agents and linear alkyl benzene (LAS) contaminating the soil and aquatic fauna [26]. These noxious chemicals also enter the human body through clothes and causes various skin and lungs diseases [27]. Thus, a development of environment friendly sustainable industrial technologies is of utmost importance considering rapid industrialization, depletion of non-renewable energy sources, increasing global energy demand, etc. In this regard, the main objective of researchers is to use enzymes as an alternative to chemical processes for the betterment of human life and to develop enzyme-based green technologies [28].

1.9.1. Freshwater mud eel gut bacterial enzyme: Protease

One of the most widely used industrial enzymes are proteases that catalyses the hydrolysis of peptide bonds in proteins [29]. In the living system proteases plays a vital role in various metabolic and physiological functions like apoptosis, cell division, blood coagulation, signal transduction, and protein catabolism [30, 31]. Although proteases can be derived from plants and animals, microorganisms are the major producers due to their easy genetic manipulation, high product yields and cost-effective production [32]. 60% of the global enzyme market is mainly comprised of microbial proteases due to their great potent applications in various industries [31]. Although proteases are produced by various microorganisms, *Bacillus* strains are often preferred as the main source of commercially

available protease due to their ability to produce huge amount of effective enzymes [33]. They are used in various sectors *viz.,* pharmaceutical, detergent, textile, leather, food, waste management, aquaculture, and others [34]. The range of protease utilization in the industries is limited despite their vast application potential due to lack of commercially desirable features among the available proteases. Industrial processes are executed under harsh conditions of high temperature and pH, hence the proteases aimed for industrial applications must have certain characteristics like stability at high temperature and pH and stability towards inhibitors, metal ions and detergent chemicals like surfactants and oxidizing agents, etc. [35]. The goal of molecular biological techniques like genetic and protein engineering is to alter the sequence of a protein to develop enzymes with preferred properties and better functions. The information generated from the availability of gene sequence, and structure-function relationship support genetic engineering approaches to design new enzymes having novel properties [36]. To meet the increasing demands of the community there is a thrust to explore enzymes with better stability through genetic engineering.

1.9.2. Classification of proteases

Exopeptidases and endopeptidases are the two classes of proteases grouped on the basis of action site [37].

1.9.3. Exopeptidase

Exopeptidase breaks the peptide bond close to the carboxy or amino termini of the substrate. Based on the site of action at C or N terminus they are further characterized as carboxypeptidases or aminopeptidases [38].

1.9.3.1. Carboxypeptidases

The carboxypeptidase cleaves the polypeptide chain at the C-terminus end with the release of single amino acids and dipeptide. According to the type of amino acid residue present at catalytic site of an enzyme the carboxypeptidase has been grouped as serine, metallo, and cysteine carboxypeptidases [38].

1.9.3.2. Aminopeptidase

The aminopeptidase cleaves the polypeptide chain at the N-terminus end with the release of tripeptide, dipeptide and, single amino acids. They cleave N-terminal methionine which are present in heterologously expressed proteins but absent in many mature proteins. Aminopeptidase are found in broad range of microorganisms [38].

1.9.4. Endopeptidase

Endopeptidase break the peptide bond by recognizing specific amino acid apart from the N and C termini. These enzymes can be further divided into serine, aspartic, cysteine, and, metalloproteases depending on the catalytic site's functional group [39].

1.9.4.1. Serine Protease

Serine proteases are widely available and can be found in both cellular and acellular organisms. These enzymes catalyzed the hydrolysis of peptide bond in the centre of a polypeptide chain. One-third of the proteolytic enzymes are comprising of serine protease [40]. An acyl-enzyme intermediate is formed due to the attacks on the carbonyl group of substrate peptide bond by Ser residue present at the active site of the enzyme. The nucleophilic activity of the enzyme is determined by the triad complex of Asp, His and Ser residues [40].

1.9.4.2. Cysteine protease

Cysteine proteases are found in all living organisms. The active sites of these enzymes are comprising of cysteine residues. These enzymes have maximum proteolytic activity in the pH and temperature ranging from 6 to 8 and 50-60°C respectively. Cysteine proteases can be inhibited by oxidizing agents, while as, their activity remained unchanged in the presence of metal-chelating agents [39].

1.9.4.3. Metalloproteases

Metalloprotease are mainly zinc containing endopeptidases. However, in some cases other metal ions such as cobalt, nickel or manganese are present at the active site. They play a vital role in various biological processes such as morphogenesis, biosynthesis of bacterial cell wall, nutrients absorption, release of cytokines, cell migration and adhesion. They required zinc and calcium for enzymatic activity and for the protein structure stability [37].

1.9.4.4. Aspartic proteases

Aspartic proteases are active in acidic pH and present in all lifeforms including microorganisms, plants, and animals. These group of enzymes have attracted the attention of the researchers due to their potential role in human diseases [41]. The enzymes are comprised of about 320-340 amino acid residues with molecular weight between 35-50 kDa [41]. Structurally, β-strands are present at the bottom of the active-site cleft and have the catalytic asparatic residues. The activity of this group of enzymes are generally inhibited by pepstatin.

1.9.5. Sources of proteases

Due to their physiological significance proteases are ubiquitous in nature, being found in various sources like microbes, plants and animals.

1.9.5.1. Microbial protease

About 60% of the global enzyme markets are comprised of microbial proteases [42]. Certain characteristics viz. rapid growth, wide biochemical diversity, easy genetic manipulation, rapid production process and easy maintenance of the organisms makes microbes especially bacteria an ideal source for the production of enzyme [43]. Microorganisms produces both extracellular and intracellular proteases. Intracellular proteases play an important role in various metabolic processes such as regulation of hormone, protein turnover, differentiation etc. Whereas extracellular proteases play a key role in various industries including pharmaceutical, food, leather, detergent, silver recovery, and waste management industries [44].

1.9.5.2. Plant protease

Plant based proteases are not abundantly available in industries as enzymes intended for industrial application requires low cost and mass production [45]. However, in recent years, research in plant-based proteases increases due to their ability to remain active in wide pH and temperature ranges [46]. Some of the proteases such as bromelain, keratinases and papain are obtained from plant sources. Papain- a cysteine protease produced from latex of raw papaya fruits. It is active in wide pH range. It is used in food industry as a meat tenderizer and in pharmaceutical industry as an antibacterial, anti-

inflammatory and antifungal agent [47]. Plant protease bromelain is derived from fruit and stem of pineapples. It is used in cosmetics due to its ability to remove the dead cells by digesting its protein [48]. Plants that are used for dehairing produce keratinase enzyme. These enzymes are used to increase the digestion of wool, hair and feather keratin and are important in the processing of keratinous wastes from leather and poultry industry [49].

1.9.5.3. Animal protease

Chymotrypsin, trypsin, renin and pepsin are the most popular proteases derived from animals [41]. Chymotrypsin is activated in the pancreas by the trypsin in a multiple step process. The optimum pH range for the chymotrypsin activity ranges from 7.5-9. Chymotrypsin is broadly used in pharmaceutical and food industry [50]. Protein component of the food is hydrolysed by the trypsin present in the intestine [51]. Pepsin is an acidic protease present in the digestive fluid of the stomach, belongs to aspartic protease family (about 36 kDa) [52]. The activity of the pepsin is inhibited by pepstatin-A. It is extensively used in the hydrolysis of proteins, gelatin and collagen extraction, as an alternative to rennet, medical research [52].

1.9.6. Application of protease in various industries

Proteases are widely used as detergent additives, an ingredient of contact lens solutions, degrading agent for keratinous wastes and collagen rich wastes generated from animal slaughter houses and fish industry, respectively, biopolishing of wool and degumming of silk, meat tenderizer, ingredient of protein and infant supplements, etc. (Fig. 1.1) [38]. Widespread use of chemicals in different industries increase environmental pollution that severely affects human health. All over the world industries are looking for alternative technologies that can fulfil the rising human demands with less depletion of natural resources [53]. In this regard, researchers aim to replace these noxious chemicals with traditionally produced enzymes that are safe and eco-friendly.

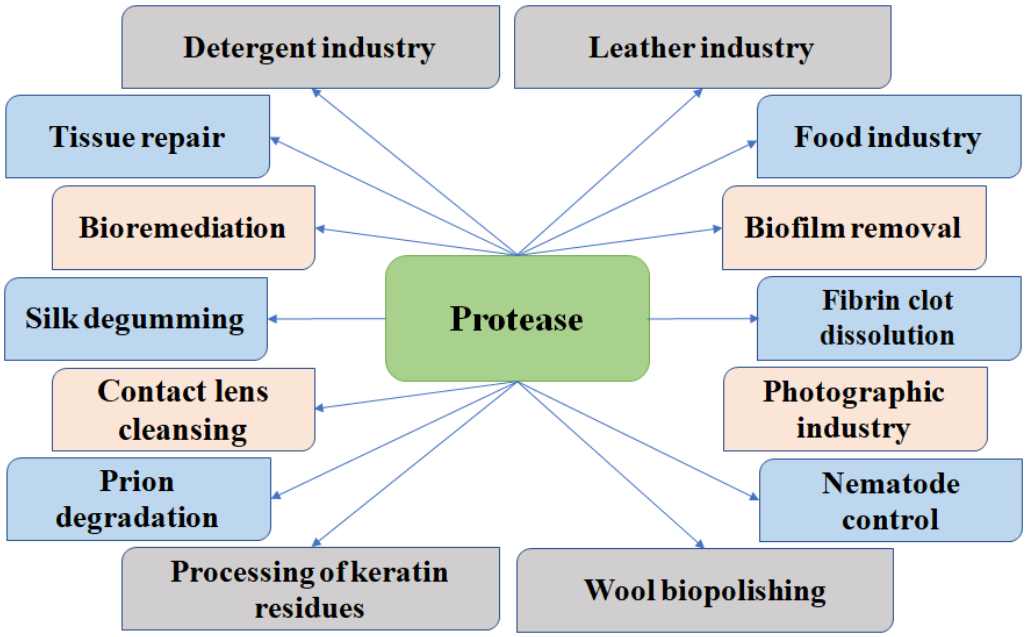

Fig. 1.1. Application of proteases in different industries

1.9.6.1. Detergent industry

Conventional detergents are mainly composed of xenobiotic compounds and hazardous chemicals such as bleaching agents, whitening agents, bluing agents, perfumers, anti-re-deposition agents and linear alkyl benzene (LAS) contaminating the soil and aquatic fauna [26]. These noxious chemicals also enter the human body through clothes and causes various skin and lungs diseases [27]. Thus, a development of environment friendly sustainable industrial technologies is of utmost importance considering rapid industrialization, depletion of non-renewable energy sources, increasing global energy demand, etc. In this regard, the main objective of researchers is to use enzymes as an alternative to chemical processes for the betterment of human life and to develop enzyme-based green technologies [28]. Application of proteases in detergent industry is a green process that act by cleaving peptide bond into small peptides present in the proteinaceous stains like blood, milk, egg, etc. [54]. Two scientists from German, Rohm and Haas first used sodium carbonate and proteases in cleaning detergents [55]. In the past 20 years, detergent enzyme market has increased almost 10-fold. Proteinaceous stains (blood, chocolate, milk and egg) are very difficult to remove by using the traditional detergent method. However, addition of enzyme to detergent formulation efficiently removes the proteinaceous stains [39]. In United states about 75% of both solid and liquid detergents contain enzymes [44]. Proteases intended for detergent industry must be stable at alkaline

pH, 60°C (wash temperature), compatibility with detergent components such as surfactants, oxidizing agents etc. [56]. Proteases isolated from various microorganisms with application in detergent industry is shown in Table 1.2.

Table 1.2 Proteases for detergent industry

Microorganisms	Type of protease	Optimum pH and temperature	Reference
Aspergillus ochraceus BT21	Serine	8 and 50°C	[57]
Bacillus megaterium TK1	Serine	8 and 70°C	[56]
Bacillus licheniformis ALW1	Alkaline	9 and 70°C	[58]
Bacillus subtilis WLCP1	Serine	8 and 15°C	[59]
Acinetobacter sp. IHB B 5011	Subtilisin	9 and 40°C	[60]
Bacillus subtilis PTTC 1023	Subtilisin	10.5 and 50°C	[61]
Bacillus pumilus MP 27	Serine	10 and 70°C	[62]

1.9.6.2. Leather industry

Traditional leather processing generates huge quantity of liquid, solid wastes (lime, and chrome dirt) and emits toxic gases such as volatile organic compounds, ammonia, hydrogen sulfide, etc. causing immense environmental problems [24, 25]. Proteases application in leather industry decreases the leather processing generated wastes causing immense environmental problems. Although various studies have done on the application of protease for dehairing, Due to some limitations such as inefficiency towards skin processing, instability in broad pH and temperature range, high cost of production etc. the tanners don’t prefer enzymes [63]. In spite of this, use of enzyme in dehairing process is

inspired due to their ability to produce high quality products with less emission of hazardous chemicals [64]. Some of the microorganism's used as a protease source in leather industry are shown in Table 1.3.

Table 1.3 Proteases for leather industry

Microorganisms	Type of protease	Application	Reference
Bacillus licheniformis	Subtilisin	Goat, Cow and Buffalo skin dehairing	[65]
Bacillus megaterium-TK1	Serine	Cow skin dehairing	[66]
Idiomarina sp. C9-1	Serine	Cattle, goat, rabbit and pig skin dehairing	[67]
Vibrio metschnikovii NG 155	Alkaline protease	Goat skin and Buffalo hide dehairing	[68]
Pseudomonas aeruginosa	Keratinase	Goat skin dehairing	[69]
Bacillus circulans	Alkaline protease	Goat skin dehairing	[70]
Bacillus cereus VITSN04	Serine	Goat skin dehairing	[71]
Aspergillus terreus 7461	Serine	Bovine leather dehairing	[72]
Bacillus crolab MTCC 5468	Serine	Goat skin dehairing	[73]

1.9.6.3. Proteases in other industries

1.9.6.3.1. Food industry

Increasing noxious chemicals in food industry exert immense pressure on food technologists to search for natural alternatives [29]. The application of microbial protease as ripening and flavouring agents effectively replaces these hazardous chemicals in food industry [74]. Application of microbial proteases could improve the flavour, nutritional and functional properties of proteins. Examples of commercial protease used in food industry include Novozym®, Alcalase®, Esperase®, Neutrase® etc. [75]. In cheese making process addition of proteases to milk aided in the breakdown of casein protein [76]. Meat tenderness is one of the dominant factors while buying meat items by the customers. Calpain 1, a calcium dependent cysteine protease is used for meat tenderization. Papain, Actinidin, proline etc. are some of the examples of proteases used in meat tenderization [77]. In baking industry, various microbes and plant-based proteases are used to degrade the gluten that is responsible for Celiac disease. Furthermore, proteases are used to hydrolysed milk for infants and to produce beer without gluten in brewing industry.

1.9.6.3.2. Pharmaceutical industry

Due to the therapeutic potential of protease, currently these enzymes are used for the treatment of various ailments. The recombinant protease Activase® was first approved to treat heart attack by US FDA in 1987 [78]. Another protease called Adagen® was first developed in1990 to treat severe combined immunodeficiency syndrome (SCID) [79]. Urokinase was formulated as replacement to surgery to cut out emboli from the occluded vessels [80]. Thrombin along with fibrin is used to treat post-surgery bleeding [81]. However, animal origin proteins are allergic in nature, therefore development of less reactive proteins like Recothrom® has been focused for treating severe bleedings [82]. Serine protease Xigris was used for the treatment of sepsis, a serious condition caused by the incursion of infectious organism in the blood [83]. Another serious condition called cystic fibrosis is treated by enzyme replacement therapy involving quantifiable combination of protease, lipase and amylase [84]. Combination of proteolytic enzymes and vitamins was formulated by Marlyn Pharmaceuticals as the brand name “Wobe Mugos” that hydrolysed the glutamyl group of folic acid and are accountable for folic acid deficiency causing death of tumour cells [85]. Moreover, FDA approved Collagenase Xiaflex to cure Dupuytren's in 2010 and Peyronie’s disease in 2013 and 2013 [86].

1.9.6.3.3. Photographic industry

Photographic waste is recycled to supply the needs of silver to the world. The traditional method of silver recovery demands the burning of films directly that give rise to undesirable bad smell and produce various hazardous emissions causing environmental pollution [39]. The chemical processes for silver recovery from X-ray films requires the use of acid and base, and hence it is completely harsh and environmentally unsafe. For these reasons, enzyme-based method for silver recovery that are environmentally safe are gaining popularity. Proteases have excellent gelatinolytic activity for effective silver recovery from X-ray films [39].

1.9.6.3.4. Silk degumming

Silk released by the *bombyx mori* are comprised of two proteins, fibroin and sericin. Structurally fibroin forms the central part of the silk that is surrounded by the sericin. Degumming is the procedure in which silk with desirable features is obtained by removing the sericin [87]. Traditionally it is achieved by using alkali or soap. However, the silk obtained by this method is of low quality, silk strength and shelf life of silk decreases [87]. Furthermore, chemicals used in traditional process causes immense environmental problems. Accordingly, there is great thrust to establish environmentally safe enzyme-based silk degumming process. Protease can break the peptide bonds of sericin without damaging the fibroin and thus could be used as a replacement to the noxious chemicals used in the process. Plant proteases (bromelain and papain) are used for cocoons processing [41].

Industrial processes based on enzymes are the most potential alternatives to costly, polluting and exhausting traditional methods. In this regard microbial proteases have been tremendously exploited and constitute the foundation for many industries. Proteases intended for commercial application must be stable under harsh industrial conditions [39]. Proteases from *bacillus* sp. has the ability to withstand harsh industrial condition such as stability at high pH and temperature, presence of inhibitors, surfactants, metallic ions, etc. Since most of the available proteases lack the industrially desirable characteristics, therefore, it is important to isolate proteases from unexplored sources with better properties. Application of proteases in detergent industry is a green process that act by cleaving peptide bond into small peptides present in the proteinaceous stains. Similarly,

application of proteases in leather industry decreases the leather processing wastes which cause immense environmental problems. In food industry, application of microbial protease as ripening and flavouring agents effectively replaces the hazardous chemicals effecting the human health. In addition to this, proteases could be effectively developed as novel therapeutic agents to overcome the cost and ill effects of the existing therapeutics and could be successfully commercialized for the development of sustainable, green and clean industrial processes.

1.10. Objectives

Based on nutritional, therapeutic and digestive enzyme value of freshwater mud eel, the following objectives were designed

1. Isolation and culture of gut microflora of *Monopterus cuchia*.

2. Morpho-biochemical characterization of pure microbial cultures and maintenance of pure cultures.

3. Purification of enzymes and their biochemical characterization with bioinformatic tools.

4. Assessment of enzymatic genes in gut derived microbial cultures as well as metagenomic (mgDNA).

5. Comparative analysis of blood and associated factors of *Monopterus cuchia* with available human blood parameters