

KERATIN BASED BIOPOLYMERIC FILM AS AN ALTERNATIVE TO CONVENTIONAL PLASTIC

Project report submitted in fulfillment of the requirement for the
degree of

Master of Science

in

Biotechnology

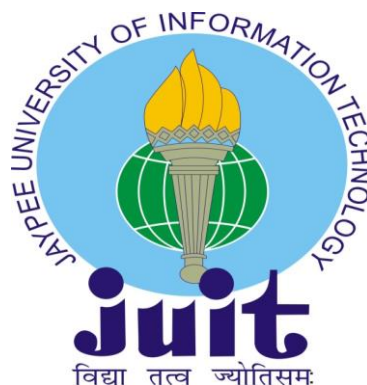
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2023

CANDIDATE'S DECLARATION

I hereby declare that the work presented in this report entitled “**Keratin based biopolymeric film as an alternative to conventional plastic**” in fulfillment of the requirements for the award of the degree of **Master of Science in Biotechnology** submitted in the Department of Biotechnology & Bioinformatics, Jaypee University of Information Technology, Waknaghat is an authentic record of my own work carried out over a period from July 2022 to May 2023 under the supervision of **Dr. Ashok Kumar Nadda, Assistant Professor, Department of Biotechnology and Bioinformatics.**

I also authenticate that I have carried out the above-mentioned project work under the proficiency stream biotechnology.

The matter embodied in the report has not been submitted for the award of any other degree or diploma.

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This is to certify that the above statement made by the candidate is true to the best of my knowledge.

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Dated:

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
Bio-PA	Bio-Polyamides
Bio-PE	Bio-Polyethylene
Bio-PET	Bio-Polyethylene Terephthalate
Bmim ⁺ Cl ⁻	Butylmethylimidazolium Chloride
CNS	Central Nervous System
-COOH	Carboxylic Acid
DCMC	Dialdehyde Carboxymethyl Cellulose
DMA	Dynamical Mechanical Analysis
DSC	Differential Scanning Calorimetry
ES	Electrospinning
FTIR	Fourier Transform Infrared
g	Gram
GHG	Green House Gas
GPTMS	3-Glycidyloxypropyl-Trimethoxysilane
H-Bonds	Hydrogen Bonds
HCl	Hydrochloric acid
HFK	Hydrolyzed Feather Keratin
H	Hour
K	Keratin
K ₂ S ₂ O ₈	Potassium Persulfate
KC	Keratin-Cellulose
KMnO ₄	Potassium Permanganate
MCC	Microcrystalline Cellulose
mg	Milligram
ml	Millilitre
mm	Millimeter
Mol	Mole
Mpa	Mega Pascal

Mt	Metric Tons
N	Normality
NaHSO ₃	Sodium Bisulfite
NaOH	Sodium Hydroxide
-NH ₂	Amino Group
OH	Hydroxide
PBAT	Polybutylene Adipate Terephthalate
PBS	Polybutylene Succinate
PEG	Polyethylene Glycol
PLA	Polylactic Acid
PVA	Polyvinyl Acetate
SEM	Scanning Electron Microscope
-SH	Thiol Group
-S-S-	Disulfide Bridge
TGA	Thermal Gravimetric Analysis
US	United States
UTM	Universal Testing Machine
UV	Ultra-voilet
Vol.	Volume
wt	Weight
XRD	X-Ray Diffraction

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ABSTRACT

This study is focused on formation of green, sustainable and cost-effective bioplastics or biopolymeric film from keratin, extracted from waste chicken feathers. Use of chicken feathers can solve two environmental problems i.e., waste management of chicken feathers and formation of alternative of synthetic plastic which can restrict environmental pollution. The chicken feathers consist around 90% keratin which is fibrous, non-combustible, biodegradable, biocompatible and insoluble in nature and classified as structural protein. It is considered as one of the hardest and most abundant protein found in the nature. Due to this chicken feather is used in this study for the formation of keratin-based bioplastic film along with glycerol, which acts a plasticizer and helps in formation of efficient and cost-effective film. Another protein, gelatin is blended with the keratin due to its film forming property. Keratin and gelatin blended formed film in this study shows good results. The keratin used in this study was dialyzed and blended with gelatin which gives thin, transparent and durable biopolymeric film. These films are characterized by using universal testing machine (UTM) and biodegradability test have been found positive. These films can be used for food packaging or bio-packaging and for other industrial applications.

Keywords: *Bioplastic, Keratin-based bioplastic, Chicken feathers, Plasticizer, Bio-packaging.*

CHAPTER-1

INTRODUCTION

Now a days, the concern to protect environment is increased due to the depleting condition of environment. Soil pollution, air pollution, water pollution (ground water, rivers and ocean) are some types of environmental pollution, that are alarming and can be hazardous for next generation. The excessive use of synthetic product like plastics, fertilizers, synthetic fibres/clothes *etc.*, and exploitation of fossils fuels are some main reasons that are behind this. Synthetic plastic is one of the leading problems of living world and the reason behind this are its non-biodegradable nature, over production, excessive dependence and poor waste management system which causing water and soil pollution. Single use plastics is especially posing the main threat to environment due to the one-time use characteristics which ultimately throw as waste material. It is found in studies that, around 50% of plastics garbage produced in 2015 was only single-use plastic, which is frequently utilized for plastic packaging and items that are being used once before being discard [1] and in studies it is also found that when utensils made up of plastic get heated along with meals it releases toxic chemicals like bisphenol-A that can lead to harmful diseases like cancer and hormonal disbalance.

Recycling of such plastic can be a short-term solution but it is not feasible enough to tackle this problem because there are various obstacles that comes during plastic recycling like separation of mixed, coloured, multi-layered plastics and difficult-to-remove residues which having unprofitable markets. Thus, only 10% of total of plastics get recycled, roughly 10% are burned, and roughly 80% are disposed of in landfills [2]. Approximately 3% among all plastics of market wind up mostly in ocean, with microplastics accounting for up to 36% of this total [3]. Around 6 billion Mt of waste plastic have formed globally, leading to serious environmental issues and concerns [2]. So, to avoid and tackle this problem green and naturally derived bio-based product are needed like bioplastics which are sustainable and environmentally friendly and can be a potential alternative of synthetic plastics.

Plastics that are made up of biodegradable polymers or having natural origin or made from waste natural polymeric resources are said to be bioplastics. It can be classified as; mixed source bioplastics (bio-petroleum), petroleum-based biodegradable polymers (fossil-based), and renewable resource-based polymers (animals, plants and waste resources) [4]. Some of the examples of renewable resource-based polymers are gelatin, cellulose, starch, pectin, chitosan, agar, dextrin, alginate, polylactic acid (PLA), food waste, lignocellulosic waste, agricultural

waste, as well as animal derived keratin protein etc. that are now widely studied and utilized for the formation of bioplastic or biopolymeric film that have application in food packaging as well as in manufacturing of various product.

Bioplastics manufacturing has expanded significantly during the previous several years, which is anticipated to continue expanding significantly in the coming years [5]. The production of eco-friendly and biodegradable plastics utilizing sustainable raw resources is a new technological objective enabling effective and sustainable production. From over past decades, biobased plastics synthesised from various proteins were studied and manufactured for a variety of uses, such as creation of coating material and films for packaging industry. The main focus was on animal proteins including casein, egg albumen, whey, keratin and collagen and as well as plant proteins like soy, wheat gluten and maize zein etc [6]. In contrast to traditional synthetic films, protein derived films have good mechanical qualities, and they have better oxygen barrier capabilities at intermediate or low humidity levels than polyolefins do [7].

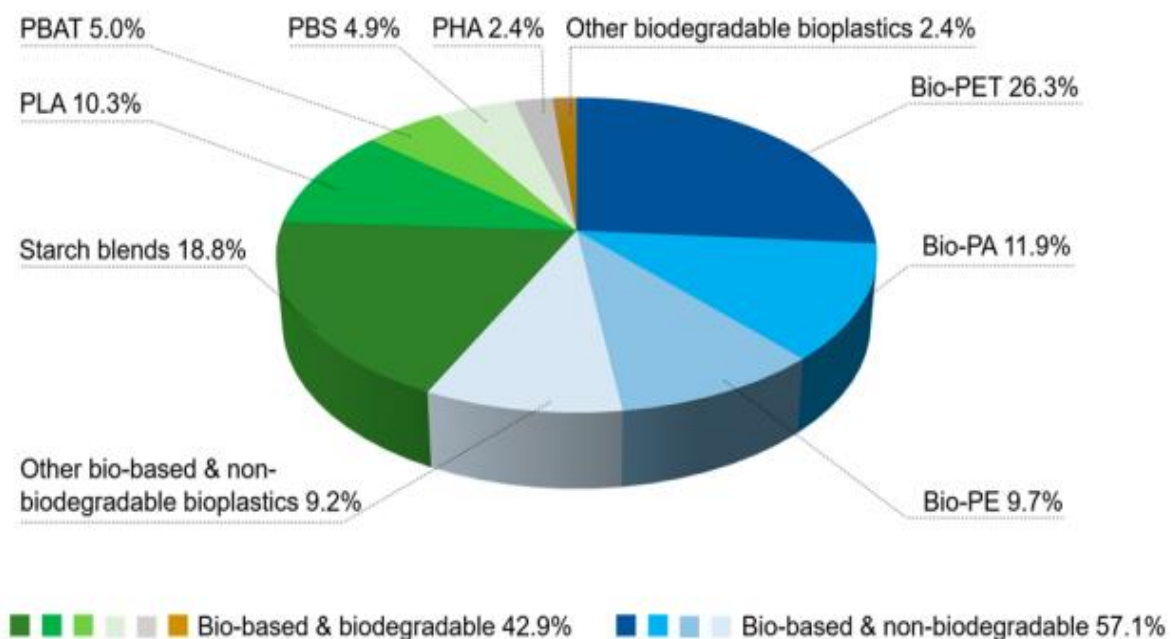


Figure 1.1: Bioplastics production potential around the world in 2017. [8]

Figure 1.1 Shows the proportion of different type of bioplastic are produced around the world, which include 18.8% Starch Blends, 10.3% of Polylactic Acid (PLA), 5% of Polybutylene

Adipate Terephthalate (PBAT), 4.9% of Polybutylene Succinate (PBS), 2.4% of other biodegradable bioplastics (bio-based and biodegradable) and 26.3% of Bio-Polyethylene Terephthalate (Bio-PET), 11.9% of Bio-Polyamides (Bio-PA), 9.7% of Bio-Polyethylene (Bio-PE), 9.2% of other bio-based and non-biodegradable bioplastics (Bio-based and non-biodegradable) in 2017.

The challenge to produce naturally derived bioplastics and to tackle the waste management system is major issue which is a worldwide known. Many researchers and scientist around the globe constantly looking for the solution to tackle this problem. One potential and possible solution is going to discuss in this study i.e., use of keratin protein from the chicken feathers which are consider as waste material and are rich in keratin. According to studies, the US chicken industry produces roughly 3 to 4 billion pounds of chicken feathers as a waste every year (Huda et al 2009) and in Canada, about 157 million pounds. The Feathers get wasted and it was hardly ever used for industrial application to make something out of it. Poultry slaughter houses all around the world have chicken feathers which is by far the most prevalent waste product. They have around 90% of keratin and can be utilized as a natural product for industrial applications in large scale for the production of keratin-based product [9][10]. Feathers constitute a significant portion of the waste produced by the chicken slaughter business. Around 5 million of tons of feathers were generated worldwide each year [11][12]. These waste feathers were dumped in the large landfills and some are combusted without taking any safety and pollution control measure which ultimately causing land, soil, water and air pollution. It is reported that, for disposing these waste feathers huge disposal space is needed, and also, they produce a greater number of pathogens or diseases, toxins, and heavy metals that are significantly bad for the environment and groundwater [13][14]. So, rather than dumping these feathers in landfills which causing environmental pollution, we can utilize these waste feathers to make or produce valuable, natural and sustainable biobased product like keratin-based bioplastic or biopolymeric film. By utilizing these waste feathers, we can produce plastic film as well as, will also be able to tackle the waste management problem caused due to chicken feathers. That's why chicken feathers are used as a source for this study to obtain keratin protein, due to their easy and high availability, low cost and its characteristics features.

The chicken feathers consist around 90% keratin which is fibrous, non-combustible, biodegradable, biocompatible and insoluble in nature and classified as structural protein. It is considered as one of the hardest and most abundant protein found in the nature [15][16].

Overall molecular mass of feathers keratin is approximately 10500 g/mol is reported [17] and it has roughly 7% cysteine, which interacts with other cysteine monomers to generate sulfur-sulfur bonds. These cysteine linkages, also known as disulfide bridges or cysteine-cysteine cross-links, gives keratin remarkable rigidity and strength in the solid form. The feather of chicken consists 50% quill an 50% fiber by mass and both can be used to produce bioplastic film. Experiments have been conducted to surface graft synthetic polymers onto poultry feather fibres or to combine them with plasticizer to create films utilising extrusion, casting and compression moulding methods. For biotechnological applications, the keratin protein isolated from chicken feathers could be employed in many forms like films, sponges, and fibres, either alone or combined with some other biopolymers like gelatin, cellulose starch or synthetic polymers. figure 1.2 Shows the different parts of a chicken feathers in which the main rod like quill is supporting the barbs.

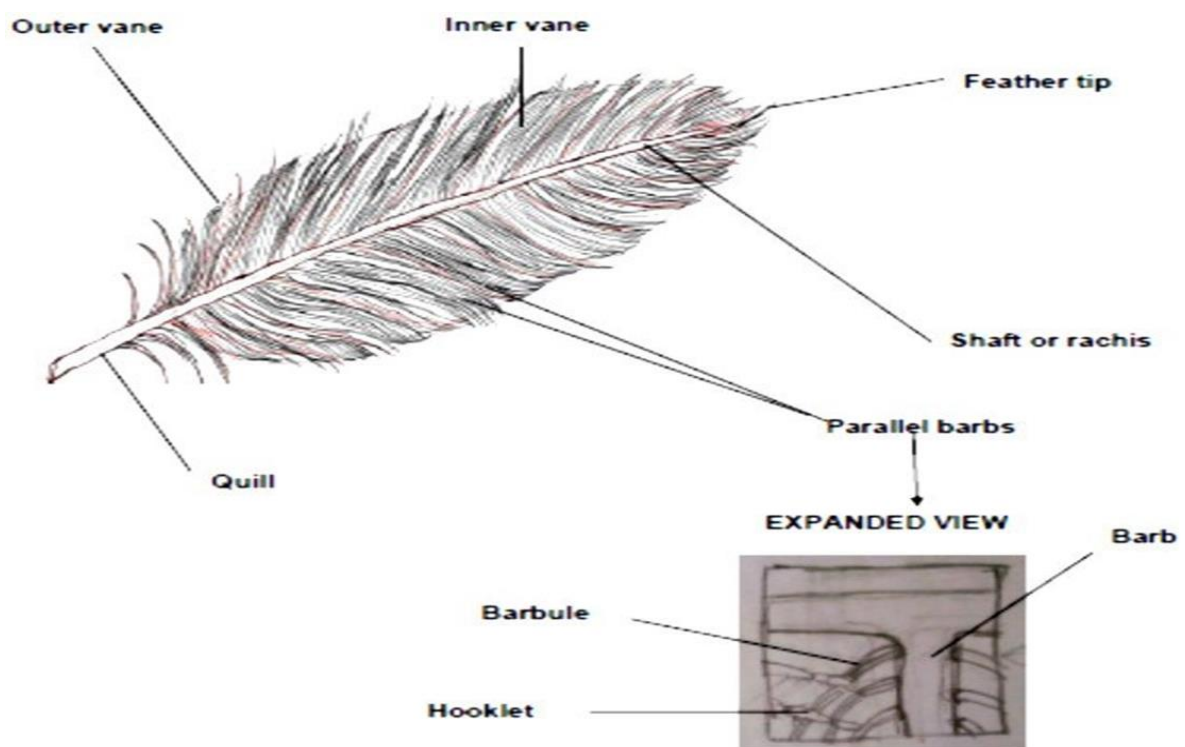


Figure 1.2: Different parts of a chicken feather. [12]

Other biopolymers like gelatin, cellulose and starch are known biopolymers that can be used for the formation of bioplastic either alone or blending with each other.

Gelatin is a protein biopolymer which is derived from insoluble fibrous collagen & is widely utilised by many different sectors due to its user-friendly and stabilizing properties. Its many benefits, including its low cost, biodegradability, polymerization, great antioxidant and antibacterial qualities, make it an excellent choice for packaging food items and because of its useful qualities, such as its capacity for forming a gel, formation of film, moisture barrier, creation of foam, and propensity for emulsification, it can be potentially and widely use in the pharmaceutical, cosmetics, photography, and food packaging sectors etc. [18].

Cellulose is a naturally occurring polysaccharide biopolymer that is widely used for the production of bioplastic due to its hydrophobic nature and high strength. A variety of sources like algae, plant, marine life, and microbes, can produce cellulose [19]. In addition to improving the biodegradable, mechanical and thermal resilience of the biopolymers, cellulose may reduce manufacturing cost [20].

This study focuses on the synthesis of keratin-based bioplastic or biopolymeric film blending with gelatin protein in which keratin was extracted from chicken feathers, which is a waste material that will be used to generate bioplastics and also it will help also help in waste management of the chicken feathers which causes environmental pollution. Both the keratin and gelatin are animal derived protein that can be bind with the help of plasticizers and crosslinking agents. Then, the glycerol solution is used and dissolved with the extracted keratin with other polymeric protein like gelatin to form a novel type of bioplastic or biopolymeric film. The glycerol is used in this study due its excellent and efficient plasticizing property. It plasticizes the protein-based polymers and it can easily penetrate into protein molecules to interact with polar groups, which ultimately modify the film characteristic and help in the formation of film. It is used in different ratio with the keratin and gelatin solution to check the plasticizing effect. In literatures it is found that ethylene glycol can also give good result as plasticizers in fact better than glycerol but in studies it is found that ethylene glycol breaks down into harmful toxic compounds in the body and can affect central nervous system (CNS), hearts and kidney which can lead to death. That's why for this study glycerol is used due to its non-toxic and nature friendly property and characteristics. After the formation of keratin-based film blended with gelatin, characterization, solubility and biodegradability of the biopolymeric film have been checked and studied.

The main objective of this research work is mentioned below which are accomplished in sequential order of their completion.

Objectives of the study

- Pretreatment and cleaning of chicken feathers using disinfecting agents
- Optimization of protein hydrolyzing agents
- Optimization of polymerizing and cross-linking agents
- Characterization of synthesized biopolymeric film

CHAPTER-2

REVIEW OF LITERATURE

The market demand of bio-based product like bioplastic or biopolymeric film is increasing due to environmental concerns and growing young population who wants to use product which is sustainable and environmentally friendly. Due to this, researchers and scientist are finding and developing new methods and green raw materials to produce bioplastics or biopolymeric film. The production of different types of bioplastics including keratin-based bioplastic, keratin blended with other biopolymers-based bioplastic, gelatin-based bioplastic and other biopolymers like cellulose, starch or food waste bioplastic are reported in literature, some of them are discussed in this chapter of this report.

2.1 Bioplastic or biopolymeric film

Sustainable packaging for food is becoming more and more popular in the food business, not simply since it shields the goods but also because it can contain biologically active components that assist to extend the shelf life of the food. Findings shows that biodegradable coatings or films derived from protein possess great mechanical characteristics and serve as an effective shield against moisture, oxygen, and microbes [21].

Bioplastics, which also known as bio-based plastics, are macromolecules of organic material that are synthesised or manufactured by humans out of sustainable biological materials including protein, cellulose, vegetable oil, microorganisms, starch etc. and are utilised in the same ways as synthetic plastics. It also stated that, despite being made using biodegradable materials, bioplastics might be biodegradable or not; this depends upon their manufacturing procedure & the purpose for which they are used [22].

Utilization of proteins to create biodegradable polymers has potential for a number of different material-based solutions [23].

Among most effective modification processes for creating thermoplastic materials using biological polymers is acetylation, which involves the esterification process by utilising acetyl anhydride [24].

In order to serve as a competitive, effective and attractive substitute to plastics derived from fossil fuels, biologically produced active packaging needs to possess excellent stability, excellent mechanical characteristics, and effective barrier characteristics [25].

The polymeric protein made up of different amino acid residues can enhance both inter- and intramolecular interactions, which can give the final products a variety of functional characteristics [26].

The manufacturing of natural and safe biopolymeric films and coatings that are edible are growing in popularity within the area of preservation of food by packaging with natural derived film, since the influence of food safety has an impact on the well-being of consumers. Due to their high biocompatibility and consequently increased technical and biological utility, biological polymers (such as polysaccharides and proteins) are frequently employed within food processing plants to create composite coatings and films [27].

Biocomposite's characteristics might be improved by interactions among fibre constituents and also the polymer matrices [28].

The performance of the films that are produced can be significantly modified when two distinct biological polymers are combined into film-forming solutions. All of these modifications depend on the molecular mass, pH, chemical composition and hydration response of the two polymers, among other factors [29].

The sustainable biopolymer derived film items and coatings have seen a significant growth in usage over the past several years in the food company's packaging needs. These materials shield dietary food items from environmental damage, lengthen the shelf life while delaying degradation, and maintain the safety and quality of the food respectively [30].

Manufacturing bioplastic addresses environmental problems because these materials are both biodegradable and recyclable in nature and can be widely utilised for different purposes [31].

One of the most popular techniques to make edible films and protective coating is solution casting method that are very cheap, effective and basic process that can create very well structure of polymers through the establishment of hydrogen bonds and intermolecular electrostatic bonds [32].

The packaging of foods has evolved into a crucial component of the contemporary food sector. Food wrapping is primarily used to create an obstacle within the environment and the food in order to give it physical protection and to minimise the contact of the food with elements that have ability to spoil it, such as atmospheric oxygen, ultraviolet (UV) radiation, microorganisms

etc. and finally to prevent from smell and flavour loss from the food with the ultimate objective to improving the food's quality and shelf life [33].

From the time the item of food is placed inside the bioplastic wrapping until the time it is removed for intake, those characteristics of bioplastic must be remained unchanged. This makes it vital that subsequent biological degradation begin immediately as the substance has been disposed, within less time [34].

Plastic polymers obtained from biomass resources have drawn significant attention throughout the bioplastic industry these days for the development of packaging materials for food and biomedical applications [35].

Despite fossil fuels, bio-based products originate through plants and animal's resources that are recyclable and renewable. Biopolymeric engaged packaging for food should ideally be capable to decompose in a simple composting environment. Technological innovation for sustainable active packaging materials holds the promise to cut back on the use of plastic derived from petroleum and other fossil fuels and the associated resulting greenhouse gas (GHG) production. Some advantages of using bioplastic as an alternative of conventional plastics made from synthetic materials involve a decrease in overall packaging's carbon impact, a decrease of waste due to plastic, as well as the preservation of fresh food items. Bioplastics have to be manufactured from sustainable or recyclable materials by using energy from renewable sources in order to develop a circular approach [36].

The packaging sector in US consumed around 28.9 million tonnes of plastic in 2005, making it among the primary uses for synthesized thermoplastic materials [37].

2.2 Keratin protein

A highly prevalent structural protein (fibrous) found in horns, bristles, hair, hooves, skin and in feathers of birds is keratin. Every year, several million tonnes of waste rich in keratin protein are produced worldwide, particularly in the woollen textile manufacturing sector and chicken slaughter houses [38].

Anticancer and antioxidant properties of keratin microscopic particles which is generated chemically from feathers of the chicken [39].

All such natural proteins like keratins do have advantageous qualities, such as bio-compatibility, biodegradability and flame-resistant capacity but even so, most of have been

typically not utilized efficiently, and also these resources have such a large (3 to 4%) sulphur content, burning and landfilling them may possess a negative effect on environment [40].

The chicken business, farms, slaughter houses, butcher shops and waste feathers, wool, nails and horns produce over 5 million tonnes of protein derived leftover each year [41].

Cross-linking is generated due to production of cystine hydrogen bonds, di-sulphide linkages and salt associations, which accounts for the integrity of keratin protein to remain in solid form [42].

2.2.1 Keratin extraction and biopolymeric film formation

When keratin was retrieved utilizing sodium sulfite, it possessed the highest antioxidant activity of all the solvents. This study added to the conversation of how functional groups, specifically -NH₂, -COOH, -SH and -S-S- interacted to free radical species once keratin has been extracted from chicken feathers. In-vitro bioactivity of isolated keratin is influenced by its molar mass, surface appearance, and crystalline nature, according to research on the impacts of extraction processes on extracted keratin. A multitude of industries, including pharmaceuticals, cosmetics, agriculture and food processing can benefit from the utilisation of bioactive antioxidant keratin [43].

Created and received a patent for making a keratin derived film from feathers of the chickens by compression molding process without the addition of oxidising or reducing chemicals and by adding at any single plasticizer that contain OH [44].

Efficiently utilized KMnO₄/malic acid redox mechanism, to modify fibre of the chicken feather with the help of graft co-polymerization using methyl methacrylate [45].

Feather of chicken was used to extract HFK (hydrolyzed feather keratin) employing a feasible combination of acid precipitation and alkali extraction and using sodium sulphide and urea. The goal was to employ a thermoplastic technique to generate and characterise environmentally friendly films derived by HFK using varying glycerol concentrations. Thermal investigation revealed that high pressure and temperature enhanced the interaction of the molecules of HFK and glycerol. Further, it was demonstrated that adding water is important for the hot-pressing of films. According to FTIR spectroscopy, novel H-bonds were formed across glycerol and HFK. It was discovered that the greatest value of elongation at break is 63.8% film using 35% glycerol [46].

The extreme sensitivity of keratin with different enzymes and chemical reagents has led to challenges throughout decades of study focused on turning crude keratin derived products to soluble phases of protein keratin. This protein is generally showing hydrophobic nature in mixtures of weak acids and alkalis, with the majority of organic liquids due to its densely conjugated network arrangement, which contains multiple hydrogen and di-sulphide bonds, in addition to interactions with hydrophobics among densely bound keratin micro fibrils. acid, Alkaline, enzyme-mediated hydrolysis, oxidation or reduction of these di-sulphide linkages, heating in some solvents that are organic, and numerous hydrothermal treatment techniques are all possible methods to obtain soluble keratin [47].

Employed $\text{NaHSO}_3/\text{K}_2\text{S}_2\text{O}_8$ as redox mechanism and grafting methyl acrylate onto fibre of native chicken feather, successfully changed the fibre and created film by compression molding method by modifying feathers of the chicken by using plasticizer like glycerol [48].

To eliminate grease from chicken feathers, 35 g of feathers were washed using petroleum ether in boiling range between 45 to 50 °C within a Soxhlet unit for 12 hours [49].

Keratin extracted from chicken feather can be used to form bioplastic. bioplastic films were made by mixing extracted keratin solution with glycerol solutions ranging in concentration from 2 to 10%. The finest thermal and mechanical qualities were found in keratin with 2% glycerol solution, which was tested by different techniques such as Thermal Gravimetric Analysis (TGA), Scanning Electron Microscope (SEM), X-ray Diffraction (XRD) and Fourier Transform Infrared (FTIR) etc. bioplastics were found biodegradable in nature [50].

According to the cyanoethylation degree, films formed out by feather that has been cyanoethylated ranged in elongation and strength from 4.2-1.6 MPa and 14%-5.8% respectively. Feathers that had been cyanoethylated had great thermoplasticity and were able to produce a variety of thermoplastic materials [51].

Cross-linked citric acid keratin films of feather derived had a substantially greater tensile strength of 230 MPa as comparison to cross-linked citric acid films [52].

Examined the thermal, water solubility, mechanical and other physical characteristics of feather keratin films using various glycerol concentrations. With the addition of glycerol at various levels of concentrations of 0.01, 0.03, 0.05, 0.07, & 0.09 gm glycerol/gm keratin, from feather keratin was recovered. It was discovered that the films' elongation at break and tensile strength both reduced. The water vapour porosity of keratin films enhanced when samples

made with 0.09 glycerol/g keratin and without plasticizer. With the addition of 0.09 glycerol/g keratin, films became more soluble. The wettability concept was said to be quite helpful in understanding the mechanisms governing the vapour barrier provided by film, that can be beneficial as a method to research on this phenomenon in multiple circumstances [53].

Chicken feather keratin film maximal tensile strength was reduced and its break elongation was raised by varying the amount of glycerol. In this manner, altering glycerol amount supplied to keratin solution used it to create the samples can be utilised to adjust elongation at break and tensile strength. These characteristics can be changed radically by modest glycerol amounts [53].

Formed keratin derived films using low keratin mixtures with varying glycerol (plasticizer) content [54].

Bioplastic film can be formed by utilizing quill of the chicken feather by using best and efficient plasticizers. Twin screw extruder was used to extrude the feather quills along with sodium sulfite solution which acts a reducing agent, after that it was dissolved in four different plasticizers for formation of bioplastic film. Ethylene glycol was found as best plasticizer followed by glycerol, propylene glycol and diethyl tartrate on the basis of Dynamical Mechanical Analysis (DMA) and Fourier Transform Infrared (FTIR) results which shows best clarity, easy processing, flowability and mechanical properties of the bioplastic film produce by utilizing Ethylene glycol [55].

2.2.2 Keratin-based biopolymeric film blended with other biopolymers

Bioplastic films have been created utilising various ratios of cellulose plus PVA/glycerol with keratin derived from feathers of the chicken. 2% of microcrystalline cellulose was added to K-60 bioplastic to create a better bioplastic (KC-60). To examine the changed film, various methods including FTIR, SEM, XRD, and TGA were employed. The TGA of K-60 and KC-60 demonstrated that cellulose and keratin cross-linked more effectively at higher temperatures. The synthesis of keratin-derived bioplastics having great strength and good crystallinity, that can be employed in biomedical field and the production of food containers among other things, was thus demonstrated by the results [56].

Smaller gaps than what the Keratin-60 are seen on the Keratin+cellulose-60, which may because of the extensive creation of intermolecular bonds [57].

By mixing glycerol (plasticizer) and edible DCMC (Dialdehyde Carboxymethyl Cellulose) with keratin extracted from feathers of the chickens, edible food packaging bioplastic film can be formed. A reduction in the tensile strength of feather keratin film with increasing DCMC content was observed which shows plasticization, brought on by DCMC's hydrophilic characteristics, partially mitigated the crosslinking impact. A small decrease in moisture sensitivity of film was revealed by a decrease in water vapour permeability, moisture content and solubility after DCMC crosslinking, which shows DCMC crosslinking improved overall potential viability of feather keratin films which can be used in food packaging purposes [58].

A novel technique for producing keratin derived bioplastics i.e., one-pot microwave-assisted procedure of extraction of keratin followed by direct electrospinning (ES). In order to create keratin derived biocompatible and biodegradable bioplastics using ES, waste-based keratin was mixed with gelatin and cross-linked with 3-Glycidyloxypropyl-trimethoxysilane (GPTMS). The characteristics of keratin derived bioplastics can be modified by varying GPTMS and keratin concentrations, and then these bioplastics can be used in sectors like purification/filtration membranes and bio-packaging as according barrier, mechanical and thermal properties characterizations by DSC (Differential Scanning Calorimetry), TGA (Thermal Gravimetric Analysis) and SEM (Scanning Electron Microscope) etc [59].

While comparing tensile strengths of bioplastic film made of keratin (10 wt%) and palm sugar starch film (15 wt%) plasticized with glycerol was found 0.0409 MPa and 9.59 MPa respectively [60].

Bioplastic film was formed by extracting keratin in powdered form, from waste chicken feathers by dissolving it in sodium hydroxide (NaOH) solution at 60 °C for 48 hours along with 0.02% microcrystalline cellulose and 3.5% glycerol. The Young's modulus of the bioplastic film was found 1.52 ± 0.34 Mpa, Thickness was 1.12×10^{-4} mm and tensile strength was 3.62 ± 0.6 Mpa, which was tested by different technique such as Fourier Transform Infrared (FTIR), X-ray Diffraction (XRD), Scanning Electron Microscope (SEM), Thermal Gravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC). The method was found promising and effective and the bioplastic film produced can be employed in the biomedical, biopolymer and pharmaceutical industries [61].

In order to improve the mechanical strength and application range of the Cellulose/Chitosan + keratin composite film, chitosan and cellulose have been introduced to keratin. Since the majority of the butylmethylimidazolium chloride ([BMIm+Cl-]), utilised was retrieved by

water washing (at least 88%) for 3-4 times. FTIR, XRD, and SEM data, among other techniques, attest the chemical integrity and uniform distribution of Keratin, Chitosan, and Cellulose in composites. TGA and Tensile strength results show that this composite film's thermal stability and mechanical strength are significantly increased by the addition of Cellulose or Chitosan to Keratin [62].

MCC and keratin film matrices interactions supported the primary function in boosting the performance of keratin derived bioplastic. As a result, the MCC and keratin strong hydrogen affinity is primarily responsible for this association [63].

2.3 Gelatin protein

An example of another biological polymer exhibiting an excellent film-forming capacity employed in the manufacturing of biodegradable as well as biocompatible biological materials, that may serve as good replacement, is gelatin [64].

A kind of bio-polymer made up of collagen, gelatin is frequently utilised to form edible coatings due to its film forming property and characteristics for the preservation of food items like fresh cherries, tomatoes, persimmons and breads, etc. [65].

Gelatin possesses the ability to create an ecosystem of actual linkages in a system that is hybrid. It has ability to create durable, elastic and strong films if the plasticization procedure is correct [66].

Gelatin's chemical composition can be altered by a number of proteins and enzymes, such as tyrosinase, glutaminase (MTGase, which functions best at 5 to 8 pH) and zein, among others, which ultimately changes gelatin mechanical characteristics [67].

Collagen was partially hydrolysed by acid or base treatment to obtain gelatin which is obtained from skin and bones from different organism which includes cattle, pigs, fish etc. [68].

Gelatin represents a protein that occurs naturally, which dissolves in water and is distinguished from other proteins that are water soluble through a lack of detectable smell & the arbitrary arrangement of chains of polypeptide in a water-based solution. It is produced by partially hydrolysing the fibrous protein collagen, that is mostly present in the connective tissues, skin, tendons and bones of both invertebrate and vertebrate organisms. In accordance with the approach of being processed, gelatin could be divided 2 two categories: category-1, gelatin having pH 8 to 9 isoelectronic point value, based on collagen that has been treated by acid; and

category-2, having 4 to 5 pH as an isoelectronic point, developed via a precursor that has been treated by an alkali (changes glutamine and asparagine amino acids to their respective acids) that produces gelatin which shows high viscosity. Typically, category-1 gelatin is made from skin of pig, while category-2 gelatin is made from skin of pig and beef, or from cattle, pig bones and hides [69].

Due to gelatin's excellent gelatinous and film-forming characteristics, modest smell, and great barrier characteristics, it can be frequently utilised for packaging of food items [70].

The animal derived protein gelatin has minimal mechanical stiffness and it is also susceptible to water vapour droplets although it exhibits high gas barrier traits and a swelling propensity in water also [71].

2.3.1 Gelatin based biopolymeric film

Gelatin protein draws a lot of potential because it is inexpensive as well as simple to generate. The key drawback of film based on gelatin is their high-water permeability due to the fact that they are hydrophilic in nature [72].

It has previously been suggested to use gelatin derived films and coatings that are edible to preserve, protect, or to enhance the foods life span or shelf-life. The chemistry of food, food organoleptic qualities, controlled release mechanisms, combined toxic effects, distribution and storage, the mechanical and physical characteristics regarding materials for packaging, and the rules to be implemented in this system's structure are all aspects that must be taken into account while building a such type of system [73].

2.3.2 Gelatin based biopolymeric film blended with others biopolymers

A method to develop a film made of gelatin and chitosan to serve as recyclable and efficient packaging films. Gelatin and chitosan were combined in order to enhance certain physicochemical characteristics. Boric acid was used to crosslink the mixture of gelatin-chitosan films in order to boost their strength, water vapour permeability, light barrier characteristics, total solubility and moisture content (%). Such produced films turned out to be both homogenous and transparent, showing excellent compatibility between various elements, according to investigation of X-ray Diffraction, optical microscopy, scanning electron microscopy, & transparency. Results showed that produced films made from gelatin and chitosan had UV-light barriers characteristics. On cross-linking, films' tensile strength improves and their moisture content (%), water solubility and water vapour permeability

decreased. Polyethylene glycol (PEG) has been employed to serve as a plasticizer in order to render these cross-linked films as more elastic while increasing their transparency as well. This investigation shows that these gelatin and chitosan based biodegradable films are effective for usage as a packaging film [74].

A method to form gelatin-based film by extrusion method. They blended deionized water, glycerol, Tween 80 and beeswax into the produced gelatin/starch combination for an additional 10 minutes, after mixing gelatin in lake over 5 minutes at room temp. The barrel that corresponded to die the extrusion process had been set at 90 °C, 100 °C, 105 °C, 110 °C, 100 °C, and 90 °C, and the rotation speed of the screw had been set at 125 rpm. extrudates had been divided into smaller pieces prior being blown onto film, and they were then kept at a fixed 23 ± 2 °C and $53 \pm 2\%$ relative humidity till 72 hours [75].

By utilising electrostatic spinning approach, they synthesised nanoparticles of chitosan with clove oil plus a gelatin-protein hybrid film for storing cucumbers. They also measured the presence of *E. coli* onto yellow blossoms under variable temperatures. The findings demonstrated that chitosan and gelatin nanoparticles with clove oil nanofiber coatings outperformed unadulterated films of gelatin in terms of their ability to inactivate antibacterial agents under lower temperatures. foods that were included in the group participating in the experiment had better look and sensory ratings during four days of preservation than the foods in the unaltered control category. When compared to the control category, taste and colour both get improved [76].

On addition of various amounts of polyphenols from tea into edible coatings and films made of gelatin and sodium alginate boosted the antioxidant properties, overall shape and architecture of gelatin-based films. Low-molecular-weight polyphenols from tea can be incorporated effortlessly into the interconnected structures of sodium alginate and gelatin, causing covalent binding that alters the mechanical characteristics of films made of gelatin, tea polyphenols and sodium alginate. The moisture permeation of gelatin and sodium alginate biopolymeric films with polyphenols from tea decreased (0.8 to 2.0%) notably when compared to standard gelatin and sodium alginate biopolymeric films, who might be related to the complicated framework created by the tea polyphenols' mosaic invading forces which lowers the permeability of water vapour. Furthermore, at 2.0% polyphenols from tea, the film's resistance to light and anti-oxidant properties are substantially enhanced in order to inhibit the

oxidation of fats and to increase the freshness or life span of food items while being stored [77].

A method of gelatin and curcumin film formation. By using the solution casting process, they created a composite biopolymeric film made up of gelatin and curcumin that contain gelatin and curcumin in 0.4 wt% and 0.02 vol% respectively. They next examined the composite biopolymeric film's resistance to oxidation property and pH receptivity. It was discovered that the biopolymeric film displayed excellent antioxidant properties and pH sensitivity and concluded that the created film can be employed as innovative packaging for food since it can assess the media's pH passively to detect spoiling of food and because the developed film has antioxidant capabilities also which will ultimately help in increase in the food's shelf life [78].

Gelatin may serve as a useful bio-polymer in the synthesis of natural derived plastics. Like a single substance, it is hydrophilic and has very low tensile strength, but it is also highly biodegradable, which makes it useful in the formation of plastics. However, for such purpose of strength, it is advised that it can be utilized in combination with gelatin polymer in order to satisfy all quality standards for the packaging applications. In results it is found that in ambient condition shelf life of apple get increased after coating with gelatin film. Incorporating essential oil like lemon grass oil with gelatin film enhanced the shelf life and properties of the film and also making it antioxidant and antimicrobial in nature. The degradation rate of the film during biodegradability test was found 14% and 16% in coastal soil and garden soil respectively. Swelling nature of the film have been found in studies also. In Thermo Gravimetric Analysis (TGA) it is found that the gelatin film gets degrade around 340 °C and sample weight get decreased as the temperature of TGA increased. Due to the biopolymer sector have a promising future, various industries today want a biodegradable, sustainable and environmentally safe polymers and for this reason these naturally derived bioplastic film can replace conventionally used synthetic plastics [79].

They created a thin biopolymeric film with minimal water vapour permeability and solubility by mixing casein, cassava starch, sorbitol and gelatin. The wrapped fruit's chlorophyll breakdown rate was slowed by preserving more of the fruit's quality and increasing its Vitamin C and soluble solid amount. Additionally, this film's application increased the lifespan of the fruit guava by 2 days. Following nine days of preservation, coated fruit continued to be green, but after just three days, untreated fruit guava loses its green colour.

From this result it became clear that film made up of gelatin-based mixtures effectively postponed ripening of food and deterioration [80].

A method of formation of gelatin and chitosan based blended film. Equal amounts of gelatin and chitosan were incorporated into a water-based solution of acetic acid (1%), stirred, heated to cause dissolution, then cooled down to room temperature. Cinnamon essential oil (2 wt%) and Tween 80 was dissolved into distilled water with vigorous stirring, the solution that will form membrane were dissolved and stir with rutin (a biopolymer-based substance) (1 wt%). A clean glass plate (Teflon film-coated) measuring 24 cm x 30 cm was cast with the film-forming solution, which then leaved at room temperature over 48 hours for drying before being peeled off film. After the results, it was concluded that synthesized chitosan and gelatin-based blended functional films have potential antioxidant and antibacterial property also, that can be used for the food packaging applications [81].

The major objective of employing organic ingredients to delay breakdown processes within the protective covering is to extend the shelf-life or lifespan of food. This is accomplished by adding anti-microbial compounds into gelatin derived coatings and films in applications related to packaging of foods. In addition to thiosulphates, bacteriocins, proteins, organic acids, fungicides, enzymes, parabens, antibiotics, isothiocyanates, metals, chelating elements, and spice extracts, an extensive list of compounds having anti-microbiological ability have been additionally suggested [82].

If gelatin and chitosan were combined, amount of beta-folding dropped but the amount of alpha-helix and arbitrary curl get high while the dissolving ability of the resulting film considerably reduced. By the incorporation of chitosan, its value of contact angle raised which increases the hydrophobicity of the surface. The relationship among polar groups and their interactions, notably prevents the movement of the groups with polarity onto their surfaces revealed via fourier transform infrared analysis (FTIR), might have been responsible for the film's improved hydrophobicity. Elongation at break and the degree of tensile strength both enhanced with chitosan concentration. Furthermore, E. coli was effectively inhibited by the chitosan-gelatin hybrid membrane [83].

A method to develop a novel type of gelatin hybrid film with Pecan walnut and Pecan Walnut Shell. For this, about 30 millilitres of an equal parts ethanol to water ratio solution, 1.2 grammes of dry gelatin and 0.24 grammes of sorbitol used as plasticizer have been added to create basic film-forming solution. After that, the mixture was subsequently stirred over 10

minutes at 40 °C to create a uniform mixture. Following that, various amounts of Pecan walnut extract were added in the following amounts: 30, 20, and 10 millilitres (resulting in final % into the film creating solution of 50, 30, and 10%), which correspond to 15, 9, and 3% of the initial dry Pecan walnut, accordingly. Just ten percent from extracts (3%) was used for the films made up Pecan Walnut Shell films since greater quantities had negative functional effects. Ethanolic solution was added to the solutions in volumes up to sixty millilitre and swirled for 15 minutes. Then, para film paper was used to cover the solutions as they were spread out on 23 cm by 15 cm plates. These had been air-dried for five days at normal room temperature [84].

A method to integrate nanoliposomes a cinnamon essential oil into gelatin films extracted from fish. After the formation of film, the 3rd and 30th days storing was used to evaluate the films' antimicrobial activity stability via the method of disc diffusion respectively. The resulting biopolymeric film having cinnamon essential oil nanoliposomes had a greater zone of inhibition than the gelatin having cinnamon essential oil did, suggesting enhanced antimicrobial persistence and a lower rate of release following 30 days of storage [85].

A method to develop a gelatin film infused with papain. In order to retain the intended quality of the bread, a papain-infused film of gelatin was created, which enhanced the quality of the refrigerated dough bread and also increased its volume. Over 2 months of storage in refrigerator, the number of thiol group molecules (-SH) as well as the level of depolymerization within the macropolymer glutenin both considerably decreased and find that there were no notable variations in stability of secondary structure during high temperature which ultimately delayed the harmful degradation of the refrigerated dough and it was concluded that frozen dough might be efficiently protected by papain and gelatin film [86].

2.4 Others biopolymers and waste-based bioplastic or biopolymeric film

Cellulosic substances are being produced in nano & micro dimensions, that augment their high crystallinity, low molecular mass, renewability and biodegradability [87].

A method to develop bioplastic from vegetable waste. By diluting conc. Hydrochloric acid, a 5% (w/w) water-based solution of HCl was created. After that, under continuous stirring, the powders obtained from waste vegetables have been dissolved in the solution of hydrogen chloride having 50 milligrammes of waste vegetable powder per 1 millilitre of solution. Over a hotplate which was adjusted at 40 °C, the vegetable mixture underwent stirring in order to prevent variations in temperature. A 3500 MWCO membrane was used to dialyze the thick

mixture for a duration of 72 hrs. over MilliQ water for 12 hrs., and it was subsequently poured onto a petri plate for drying over a minimum of 48 hrs. at room temperature [34].

Waste obtained from food can be a great natural and sustainable source of polymers that are capable of being used to make bioplastic. This originates in food manufacturing sector or comes from domestic use, like feathers of chicken, sludge waste, seeds of durian, banana peels, jackfruit seeds, cassava peels and seeds of avocado etc. Subsequently lowering both food and plastic waste together, the creation of bioplastic using food leftovers or waste has the potential to address pollution issues & achieve the sustainability of the environment also [88].

One of the primary limitations when employing starch for bio-thermoplastics involves the possible negative influence upon the cost of food triggered by the rise in starch consumer demand. It is now becoming very crucial to keep the prevailing cheap price & ample accessibility to starch because it has become a highly prevalent source of natural carbohydrates throughout the diet of humans & can be found throughout in numerous everyday food items worldwide. Rising thermoplastic starch and dietary starch use could result in an inadequate supply of starch for the food sector and also higher pricing for starches-rich crop products as the number of people worldwide continues to increase [89].

CHAPTER-3

MATERIALS AND METHODS

Present report is on formation of “Keratin based biopolymeric film as an alternative to conventional plastic”. For this keratin was used as natural biopolymer for synthesis of bioplastic or biopolymeric film. The solubility, biodegradability and mechanical properties of the biopolymeric film were studied after the synthesis.

- **Experimental site**

All the experiments for formation of keratin-based biopolymeric film were carried in the genomics laboratory of department of biotechnology and bioinformatics at Jaypee University of Information Technology.

- **Experimental material**

keratin was used as sole material for this study, which was extracted from chicken feathers collected from Modern Slaughterhouse, M.C Abattoir, Shimla, Himachal Pradesh. Keratin used in this study were extracted in batches in laboratory condition. which is present in bulk for further experiments for formation of biopolymeric films. This study focusses on extraction of keratin protein from chicken feathers and formation, optimization and characterization of biopolymeric film.

Gelatin powder was purchased from Loba Chemie Pvt. Ltd and dialysis membrane 70Av was purchased from Himedia/LA393-1MT.

3.1 Pretreatment and cleaning of chicken feathers using disinfecting agents

Chemicals required:

- Petroleum ether/hydrogen peroxide (to remove blood stains).
- Labdet detergent.
- Water

Procedure:

- Chicken feathers were collected from slaughter house.
- Remaining skin residue have been removed from feathers and washed with normal tap water for 2-3 times.
- Feathers were soaked in detergent (labdet) with water for at least for 2 h.
- Boiled at 90 °C for 1 hr

- After that, soaked in petroleum ether or hydrogen peroxide
- Washed 2 times with normal tap water.
- Dried for 2-3 h in direct sunlight or in the incubator at 40°C.
- After drying, properly packed in plastic seal bags and stored them for further analysis.

3.2 Extraction of keratin and optimization of protein hydrolyzing agent (NaOH)

Chemicals and equipment's required:

- Chicken feather
- Distilled water
- Sodium hydroxide (NaOH) pellets
- Hydrochloric acid (HCl)
- Glass wares (beakers, conical flask, glass rod)
- Plastic sieves
- Weighing balance
- Mechanical overhead stirrer
- pH meter
- fume hood
- Incubator
- Lyophilizer
- Grinder
- Centrifuge

For the extraction of keratin from chicken feathers alkaline hydrolysis method is used.

Procedure:

- 300 gm of pretreated chicken feathers are chopped in small sizes.
- Dissolved in 6 L of NaOH solution by a mechanical overhead stirrer for 2 h at 500 rpm.
- Continuous shaken for 6 h at 40 °C at 150 rpm in the incubator.
- Filtered the lysate with the help of plastic sieve.
- Pure dissolved lysate is obtained.
- Precipitation was done under fume hood with (2N HCL) by maintaining pH up to (5-6) with the help of a pH meter.
- Precipitates were washed 3 times with distilled water to remove NaOH and hydrochloric acid.

- Centrifuged to get the keratin pellets.
- Freezed dry at -55 °C for 24 h by using the lyophilizer to obtain the keratin powder.
- Stored at 4 °C for further analysis.

Yield of the keratin is calculated by using this formula: $\text{Yield} = (\text{Weight of the keratin} \div \text{Initial weight of biomass}) \times 100$.

For the optimization of hydrolyzing agent 5g chicken feathers were dissolved in different concentration of NaOH solution of 100 ml under different condition.

- 0.1 N
- 0.2 N
- 0.4 N
- 0.6 N
- 0.8 N
- 1.0 N
- 1.2 N
- 0.5 N in dark
- 0.5 N in microwave

3.3 Synthesis of biopolymeric film and their optimization

3.3.1 Gelatin based biopolymeric film

Chemicals and equipment's required:

- Gelatin
- Glycerol solution (0.4%)
- Baking sheet or parchment paper
- Distilled water
- Beakers (250 and 500 ml)
- Measuring cylinders (10 and 1000 ml)
- Petriplates
- Glass rod
- Magnetic bead
- Hot plate with magnetic stirrer
- Weighing balance

To make 0.4% glycerol solution, 2 ml of pure glycerol is mixed with 500 ml distilled water.

Procedure:

- 180 ml of 0.4% glycerol solution was taken in a 250 ml beaker.
- The solution was heated on hot plate at 50°C with continuous stirring (magnetic stirrer) without boiling it for 1 h.
- After the solution get heated, 3.375 g gelatin was added and set the hot plate at 90°C for proper mixing.
- After mixing, poured the solution in a petri plate by forming thin layer.
- Leaved the petriplate for air dry for 2 days at normal room temperature.
- After 2 days, the thin bioplastic film was formed and it was removed from the petriplate carefully.

3.3.2 keratin based biopolymeric film blended with gelatin

Materials and equipment's required:

- Dialyzed keratin solution
- Gelatin
- Glycerol solution (0.5%)
- Baking sheet or parchment paper
- Distilled water
- Beaker (250 ml)
- Measuring cylinder (100 ml)
- Petriplates
- Glass rod
- Magnetic bead
- Hot plate with magnetic stirrer
- Weighing balance

Keratin solution used in this experiment was dialyzed for 4 days to remove NaOH by using dialysis membrane.

Procedure:

- 90 ml of 0.5% glycerol solution was taken in a 250 ml beaker.

- The solution was heated on hot plate at 50°C with continuous stirring (magnetic stirrer) without boiling it for 1 h.
- After the solution get heated, different amount of gelatin and dialyzed keratin solution was added in the solution and set the hot plate at 90°C for proper mixing.
- After mixing, poured the solution in a petri plate by forming thin layer.
- Leaved the petriplate for air dry for 2 days at normal room temperature.

3.3.3 Optimization of biopolymeric film

The optimization of polymeric film was carried out in 3 sets of experiments:

- Set-1: Different concentration of set of glycerol (0, 0.5, 1.0, 1.5, 2.0 and 2.5 %) was taken with constant amount of gelatin (1 g) and dialyzed keratin (10 ml).
- Set-2: Optimized glycerol solution (0.5%) and constant amount of dialyzed keratin (10 ml) was taken with varying amount of set of gelatin (0, 0.2, 0.4, 0.6, 0.8 and 1.0 g).
- Set-3: Optimized glycerol solution (0.5%) and constant amount of gelatin (1 g) was taken with varying amount of set of dialyzed keratin (0, 5, 10, 15, 20 and 25 ml).

The chemicals, equipment's and procedure for this experiment are same as followed in section 3.3.2.

3.4 Characterization of the synthesized keratin-gelatin blended biopolymeric film

3.4.1 Solubility test of the biopolymeric film

Material required:

- Keratin-gelatin blended film (3:2)
- Normal tap water
- Petri plate

Procedure:

- Normal tap water taken in a petri plate
- Synthesized biopolymeric film have been soaked in petri plate
- Time duration of solubility of the film have been recorded.

3.4.2 Biodegradability Test

The biodegradable nature of the synthesized biopolymeric film have been checked and for this analysis, film was put in set of environmental condition (in-vitro) and observation was recorded:

- Control – film was put in a beaker
- Water – film was submerged in a beaker having tap water
- Wet soil – film was put in wet soil sample in a beaker
- Daily wet soil – film was put in soil sample in a beaker with daily watering
- Dry soil - film was put in dry soil sample in a beaker

3.4.3 Mechanical properties

The thickness, elongation and tensile strength of the keratin-gelatin blended biopolymeric film have been characterized from CIPET-CSTS Baddi with the help of Universal Testing Machine (UTM) facility.

The result and data of all the above sections i.e., 3.1., 3.2., 3.3. and 3.4. are discussed in detail in chapter 4 respectively.

CHAPTER-4

RESULTS AND DISCUSSION

4.1. Pretreatment and cleaning of chicken feathers using disinfecting agents

In order to obtain keratin protein from chicken feathers, it is necessary to do pretreatment of the feathers by following a set of procedure which have to be efficient, hygienic, time saving and cost effective.

For this, first the chicken feathers were collected from slaughter house and after that in laboratory condition with proper safety procedure, skin residue from feathers have been removed and washed it with normal tap water for 2-3 time in order to remove the blood. After that, it was soaked in labdet detergent with water for at least to 2-3 hours in order to decontaminate it from harmful pathogens and dust. After the decontamination of feathers it was boiled at 90 °C for 1 hr to remove the blood stains, fats, and grease from them followed by soaking the feathers in petroleum ether [49] or hydrogen peroxide for removing the left out or remaining fat and other residues. After that, the soaked feathers were washed 2 times with normal tap water in order to remove the excess chemicals. Then, the feathers were kept under direct sunlight for 2-3 hours for proper drying and after drying the feathers were sealed packed in plastic bags for further use. The steps followed for pre-treatment of cicken feathers are showned in figure 4.1.

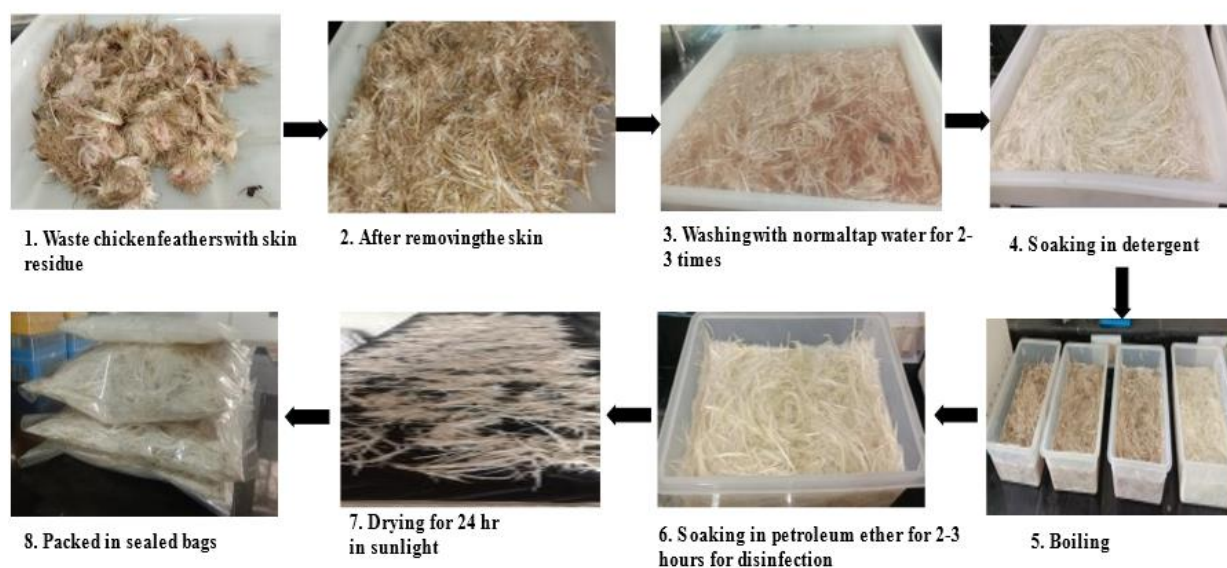


Figure 4.1: Steps of pre-treatment of chicken feathers.

4.2 Extraction of keratin and optimization of protein hydrolyzing agent (NaOH)

The extraction of keratin was done by following alkaline hydrolysis method. The pre-treated chicken feathers were dissolved in 0.5 N sodium hydroxide (NaOH) solution for the degradation of feathers [90] to form lysate after filtration with plastic sieve which removes the undissolved part of the feathers. After that, the filtered lysate was treated with 2N Hydrochloric acid (HCl) under fume hood by maintaining the pH up to 5 to 6, which precipitated the keratin precipitate from lysate. Washing of keratin precipitate for 2-3 times was done in order to wash off the NaOH and HCl residues. To get the fine keratin powder from the keratin precipitate, lyophilizer (freeze drying) were used which removes the excess water from the keratin precipitate to extend the shelf life of the keratin which can be stored for long time and can be used for further process. The keratin extraction steps are showed in figure 4.2.

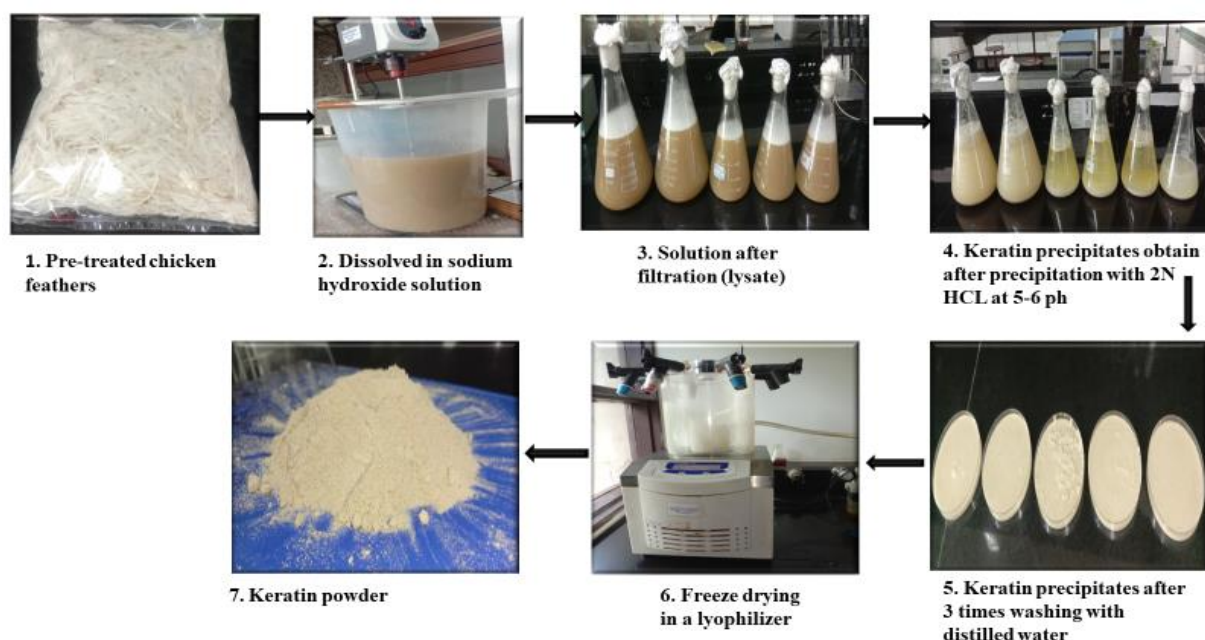


Figure 4.2: Steps of keratin extraction

In this study, sodium hydroxide (NaOH) is used as hydrolyzing agent for the degradation of chicken feather and to extract keratin protien from them. For this various concentration of NaOH under different condition is used to find and get the optimized concentration of NaOH and the condition which can hydrolyses the chicken feathers and help in extraction of keratin efficiently in less time and in cost effective manner. The result of the optimization reaction was shown in table 4.1, where 0.4 N concentration of NaOH can be said as optimized concentration because as commpared to other its takes moderate time (6 h) for hydrolysis and gives keratin yield more than 50 % by utilizing NaOH of cost around 1 Rs. only.

In microwave condition with 0.5 N NaOH is also can be used but due to high temperature and heat exposure in microwave it can cause degradation of keratin protien. So, it can be concluded that concnetration of NaOH between 0.4 to 0.6 can hydrolyze feathers more efficiently in less time and in cost effective manner.

Table 4.1: Optimization of hydrolysing agent (NaOH)

Concentration of NaOH (100ml)	Chicken feathers (g)	Temperature and shaking (° C/rpm)	Dissolution time	Keratin (g)	Keratin yield (%)	pH condition	Cost of NaOH (Rs.)
0.1 N (0.4 g)	5	50/140	4 days, partially dissolved	1.2	24	6	0.22
0.2 N (0.8 g)	5	50/140	2 days	3.2	64	6	0.45
0.4 N (1.6 g)	5	50/140	6 h	2.9	58	6	0.98
0.6 N (2.4 g)	5	50/140	6 h	2.36	47.2	6	1.34
0.8 N (3.2 g)	5	50/140	5 h	2.8	56	6	1.79
1.0 N (4.0 g)	5	50/140	5 h	2.4	48	6	2.23
1.2 N (4.8 g)	5	50/140	5 h	2.2	44	6	2.68
0.5 N in dark (2.0 g)	5	Kept in dark without shaking	2 days	2.1	42	6	1.12
0.5 N in microwave (2.0 g)	5	Microwave at 300-500	15 minutes	2.6	52	6	1.12

4.3 Synthesis of biopolymeric film and their optimization

4.3.1 Gelatin based biopolymeric film

Gelatin based biopolymeric film have been formed [79] which was plasticized by using 0.5% glycerol solution. The film formed was thin and transparent in nature with good strength. The results proved the property of film forming nature of gelatin, that's why it was used in this study to blend with keratin protein so, it can modify and add its property of film forming in keratin based biopolymeric film. Figure 4.3 shows the transparency of the synthesized gelatin based biopolymeric film.

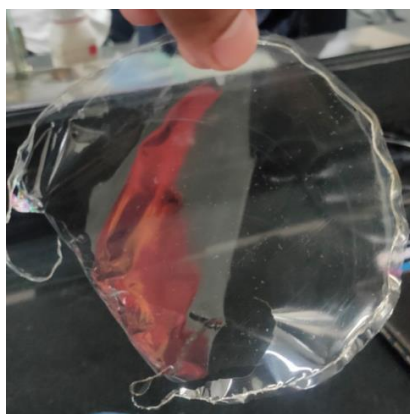


Figure 4.3: Gelatin based biopolymeric film

4.3.2 Keratin based biopolymeric film blended with gelatin

The modified method to make biopolymeric film by blending gelatin with dialyzed keratin solution in 0.5 % glycerol solution worked and give positive result. The film was found thin, transparent and strong, similar to synthetic plastic film. The transparency and improved strength of the film shows that both gelatin and dialyzed keratin get linked together [59] efficiently in presence of glycerol [54] which enables the plasticity of the synthesized keratin-gelatin based blended biopolymeric film. Figure 4.4 shows some different aspects of synthesized keratin-gelatin based blended biopolymeric film of different sizes and also showing transparency of the film.

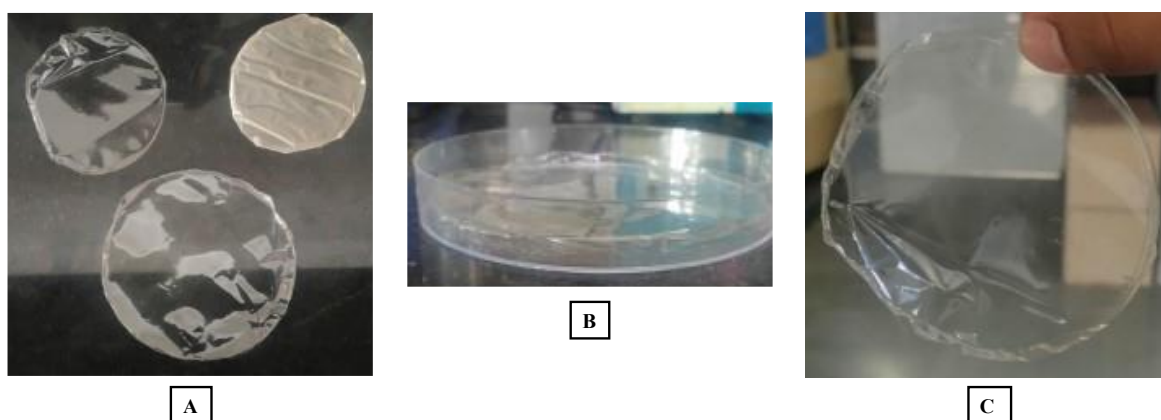


Figure 4.4: Keratin and gelatin blended biopolymeric film. A shows different sizes of films including one on a baking sheet having dark color than other ones. B is the film within the petriplate. C is the film obtained after peeled from petriplates which shows transparency of the film

4.3.3 Optimization of biopolymeric film

Optimization of biopolymeric film was achieved by doing various experiments and for this, 3 sets of experiment was done in which glycerol concentration, dialyzed keratin and gelatin amount was varied in order to find the optimized concentration of glycerol and amount of keratin and gelatin for the formation of best keratin-gelatin blended biopolymeric film which having improved and optimized characteristics that can be used for soap and food packaging applications.

- Set-1: Different concentration of set of glycerol (0, 0.5, 1.0, 1.5, 2.0 and 2.5 %) was taken with constant amount of gelatin (1 g) and dialyzed keratin (10 ml).

In this experiment, 6 set with 0 %, 0.5%, 1.0%, 1.5%, 2.0% and 2.5% glycerol solution of 100 ml were mixed with 1 g gelatin powder and 10 ml dialyzed keratin solution by following the protocol in order to get the best and optimized concentration of glycerol for the formation of keratin-gelatin blended biopolymeric film, which gives varying result showed in figure 4.5. and reported in table 4.2.

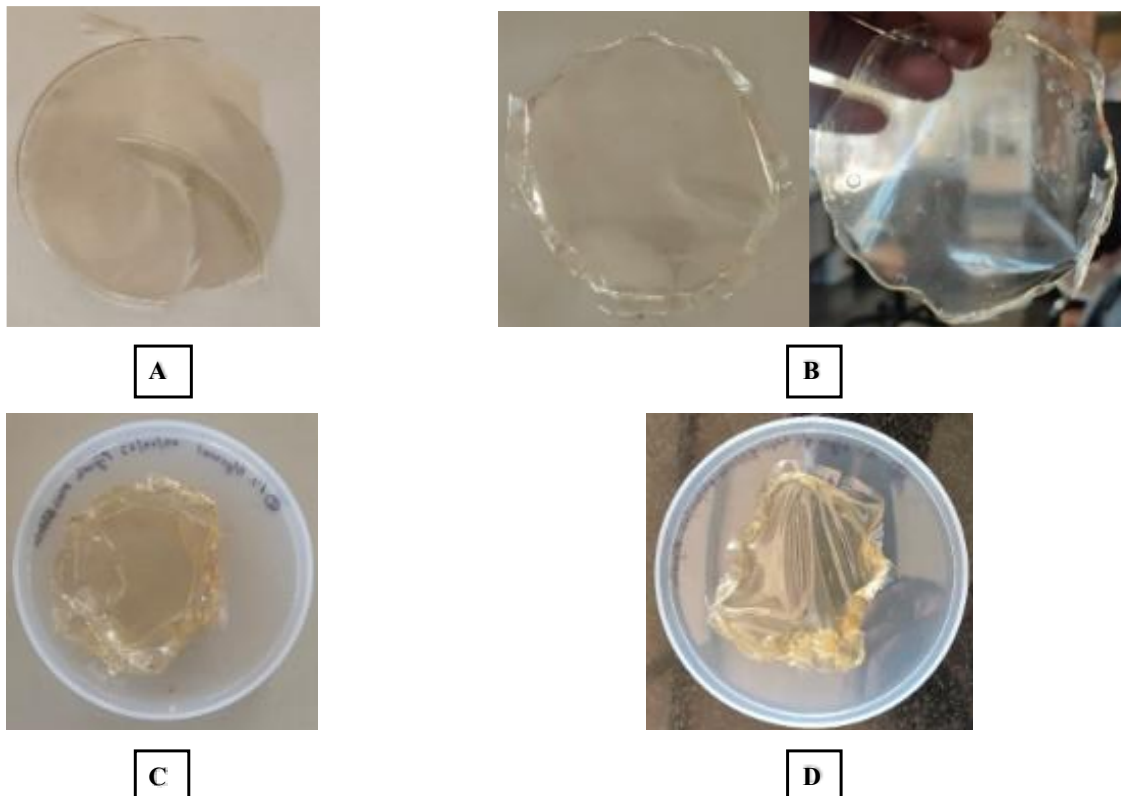


Figure 4.5: Set-1 biopolymeric film formation with different concentration of glycerol.

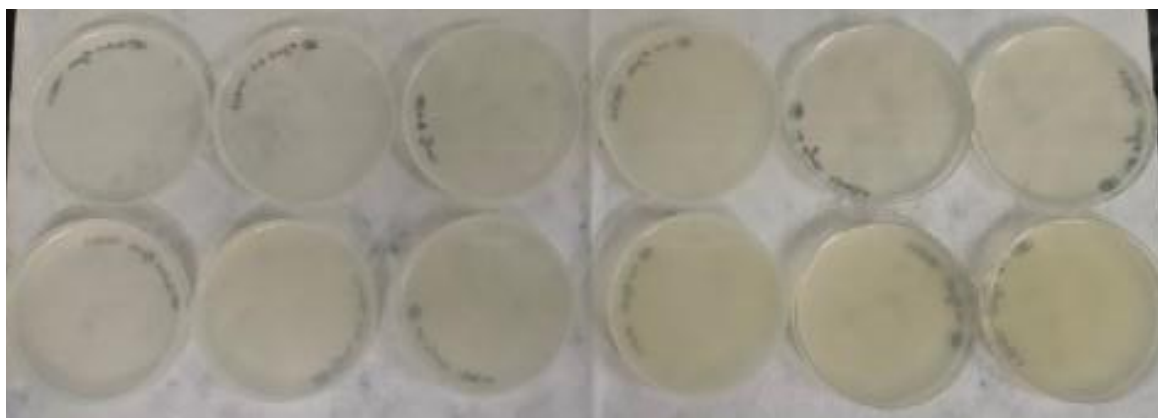
A is control, having no glycerol. B is film having 0.5% glycerol. C is film having 1% glycerol. D is film having 2% glycerol.

Table 4.2: Set-1 biopolymeric film formation with different concentration of glycerol.

S.No.	Glycerol conc. (%)	Dialyzed keratin (ml)	Gelatin (g)	Poured amount (ml, mm)	Result at room temperature (25 to 30° C)
1.	Distilled water (control)	10	1	~40, 7	The solution gets dried but the film has been formed with no plasticity and get break easily
2.	0.5	10	1	~40, 7	Film has been formed with good plasticity
3.	1.0	10	1	~40, 7	Slightly sticky film has been formed
4.	1.5	10	1	~40, 7	Sticky film
5.	2.0	10	1	~40, 7	Sticky film
6.	2.5	10	1	~40, 7	Not dried

This experiment showed, the control film formed in absence of glycerol have brittle nature, have no plasticity and get break easily [50]. Film having 0.5% glycerol was formed with good plasticity and can be bend and stretched with no sign of breaking and cracking which shows the plasticizing effect of glycerol [54] by forming hydrogen bond with keratin and gelatin but when glycerol concentration was increased from 0.5 % the resulting film getting sticky and not getting dried fully at room temperature. Only 0.5 % glycerol concentration film have been formed in time with positive result having good plasticity, good strength and transparency therefore it was considered as optimized concentration for the formation of keratin-gelatin blended biopolymeric film.

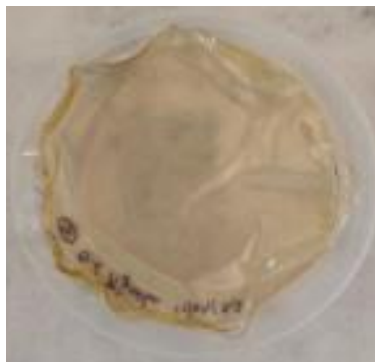
- Set-2: Optimized glycerol solution (0.5%) and constant amount of dialyzed keratin (10 ml) was taken with varying amount of sets of gelatin (0, 0.2, 0.4, 0.6, 0.8 and 1.0 g). In this experiment, 6 set with 0 g, 0.2 g, 0.4 g, 0.6 g, 0.8 g and 1.0 g gelatin powder and 10 ml dialyzed keratin solution were mixed in 100 ml of 0.5 % glycerol solution by following the protocol in order to get the adequate and optimized amount of gelatin for the formation of keratin-gelatin blended biopolymeric film, which gives varying result showed in figure 4.6 and reported in table 4.3.



A



B



C



D



E

Figure 4.6: Set-2 biopolymeric film formation having varying amount of gelatin. A is whole set-2 reactions before drying of film forming solution. B is film having 0.4 g gelatin. C is film having 0.6 g gelatin. D is film having 0.8 g gelatin. E is film having 1 g gelatin.

Table 4.3: Set-2 biopolymeric film formation having varying amount of gelatin.

S.No.	Glycerol conc. (%)	Dialyzed keratin (ml)	Gelatin (g)	Poured amount (ml, mm)	Result at room temperature (25° to 30° C)
1.	0.5	10	0	~40, 7	In absence of gelatin, film not have been formed and the poured solution not get dried at room temperature.
2.	0.5	10	0.2	~40, 7	Some amount of film formation was observed, but it was very sticky and can't be peeled off from the plates.
3.	0.5	10	0.4	~40, 7	Partially dried and sticky film have been formed.
4.	0.5	10	0.6	~40, 7	Partially dried and sticky film have been formed.
5.	0.5	10	0.8	~40, 7	Dried but film was slightly sticky
6.	0.5	10	1.0	~40, 7	Proper film has been formed with no stickiness

This experiment showed, in first reaction (control) having no gelatin doesn't get dried at room temperature and film has not been formed. In second reaction having 0.2 g gelatin, some amount of film formation was observed, but it was found very sticky which can't be peeled off from the petri plates. Third and fourth reaction having 0.4 g and 0.6 g gelatin respectively get partially dried but the film was found sticky in nature. In fifth reaction of this set, which having 0.8 g gelatin in the film forming solution get dried at room temperature in 2 days but still the film has been found slightly sticky in nature. The sixth reaction which having 1 g gelatin get fully dried and film have been formed with no stickiness in 2 days at room temperature. That's why 1 g gelatin for this set is considered as optimized amount which forms biopolymeric film with best result having transparency and good strength under same condition. It can be concluded that, for the formation of keratin-gelatin blended biopolymeric film gelatin should not be less than 1% in the final solution in order to get better result and also only in presence of keratin alone film formation from this method is not possible. This experiment proves the film forming property of gelatin [70].

- Set-3: Optimized glycerol solution (0.5%) and constant amount of gelatin (1 g) was taken with varying amount of set of dialyzed keratin (0, 5, 10, 15, 20 and 25 ml). In this experiment, 6 set with 0 ml, 5 ml, 10 ml, 15 ml, 20 ml and 25 ml dialyzed keratin solution and 1 g gelatin powder were mixed in 100 ml of 0.5 % glycerol solution by following the protocol in order to get the adequate and optimized amount dialyzed keratin for the formation of keratin-gelatin blended biopolymeric film having better characteristics. This experiment gives varying result which was showed in figure 4.7 and reported in table 4.4.

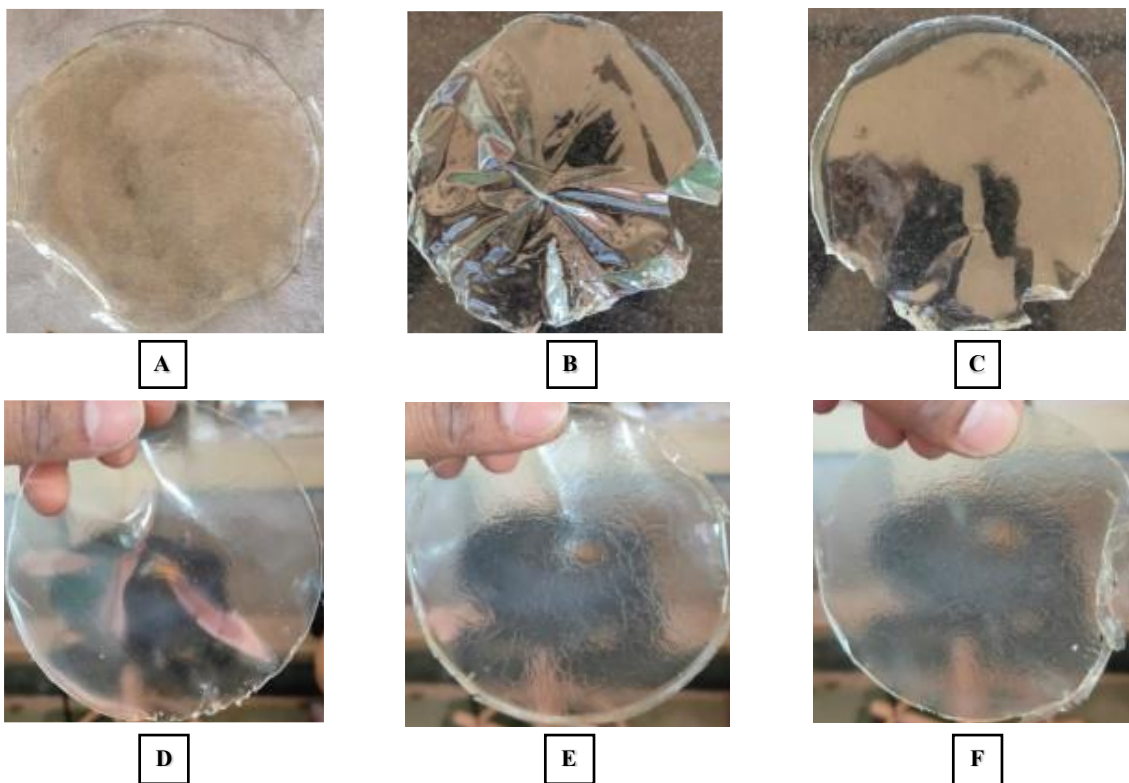


Figure 4.7: Set-3 biopolymeric film formation having varying amount of dialyzed keratin. A is control, having no dialyzed keratin. B is film having 5 ml dialyzed keratin. C is film having 10 ml dialyzed keratin. D is film having 15 ml dialyzed keratin. E is film having 20 ml dialyzed keratin. F is film having 25 ml dialyzed keratin.

Table 4.4: Set-3 biopolymeric film formation having varying amount of dialyzed keratin.

S.No.	Glycerol conc. (%)	Dialyzed keratin (ml)	Gelatin (g)	Poured amount (ml, mm)	Result at room temperature (25° to 30° C)
1.	0.5	0	1	~40, 7	In absence of keratin, film have been formed which shows the property of gelatin i.e., film forming.
2.	0.5	5	1	~40, 7	Film having 5 ml keratin have been formed with clear transparency
3.	0.5	10	1	~40, 7	Film having 10 ml keratin have been formed with good transparency, good strength and good plasticity
4.	0.5	15	1	~40, 7	Film having 15 ml keratin have been formed with slightly less transparency, good strength and better plasticity.
5.	0.5	20	1	~40, 7	Film having 20 ml keratin have been formed translucent in appearance with good strength and less plasticity.
6.	0.5	25	1	~40, 7	Brittle and translucent film have been formed with decreased plasticity

This experiment showed, the first reaction (control) having only gelatin produces a film which shows, in absence of keratin, film can be formed which refers the property of gelatin i.e., film forming. Film having 5 ml dialyzed keratin have been formed with clear transparency and the film having 10 ml dialyzed keratin have also been formed with good transparency, improved strength and good plasticity. The film formed in the fourth reaction which having 15 ml dialyzed keratin have been found less transparent but the strength and plasticity of the film was found better than the third reaction film. The fifth reaction film having 20 ml keratin have been formed translucent in appearance with good strength and less plasticity and the sixth reaction film have been formed Brittle and translucent with least plasticity. By this experiment it can be concluded, by increasing the amount of keratin, the strength [17] of the film can be gotten better but the plasticity and transparency of the film will decrease respectively. Therefore, 15 ml dialyzed keratin is considered as optimized amount for the formation of keratin-gelatin blended biopolymeric film.

The optimized film having 3 parts of keratin and 2 parts of gelatin have been shown in figure 4.8., which is synthesized by using same method having 0.5% glycerol, 15 ml dialyzed keratin which is equivalent to 1.5 g and 1.0 g gelatin.

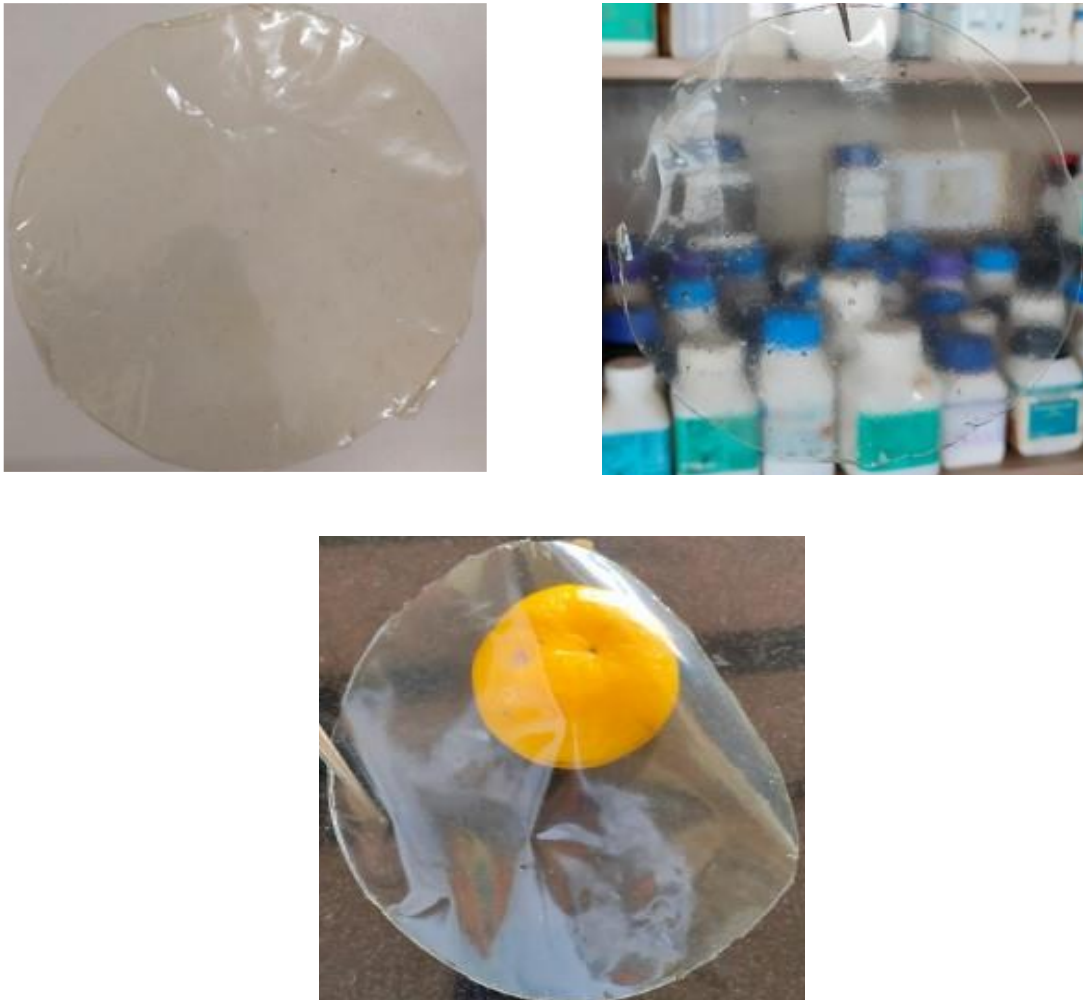


Figure 4.8: Optimized keratin-gelatin blended biopolymeric film (3:2)

4.4 Characterization of the synthesized keratin-gelatin blended biopolymeric film

4.4.1 Solubility of the biopolymeric film

In order to check the nature of the synthesized keratin-gelatin blended biopolymeric film with the water, solubility test has been done.

In results, it was found that when the film comes in contact with water, it starts retaining the moisture and water content, showing the hygroscopic nature which is happening due to the

presence of gelatin which are naturally hygroscopic in nature and get dissolved in water [69] in few minutes. Due to the insoluble and hydrophobic property [47] of the keratin, the synthesized film only gets partially dissolved in water in 4 hours of incubation at room temperature. Even after 4 days dissolution the keratin part doesn't get dissolved in water. After the test, it can be concluded that, the synthesized keratin-gelatin blended film having hygroscopic properties and are partially soluble in water which are safe and non-toxic. The before and after result of solubility test of the biopolymeric film can be seen in figure 4.9.



Figure 4.9: Solubility test of keratin-gelatin blended biopolymeric film. A is initial film before getting soluble. B is after film get partially soluble.

4.4.2 Biodegradability Test

In order to prove the biodegradable nature of the synthesized biopolymeric film biodegradability test has been performed by exposing the biopolymeric film in set of several environmental condition (in-vitro). The film was cut into 5 cm x 5 cm dimension and placed in the respective condition. The different environmental condition can be seen figure 4.10 i.e., control, water, wet soil, daily wet soil and dry soil respectively. All the observation of this experiment was recorded and reported in table 4.5.

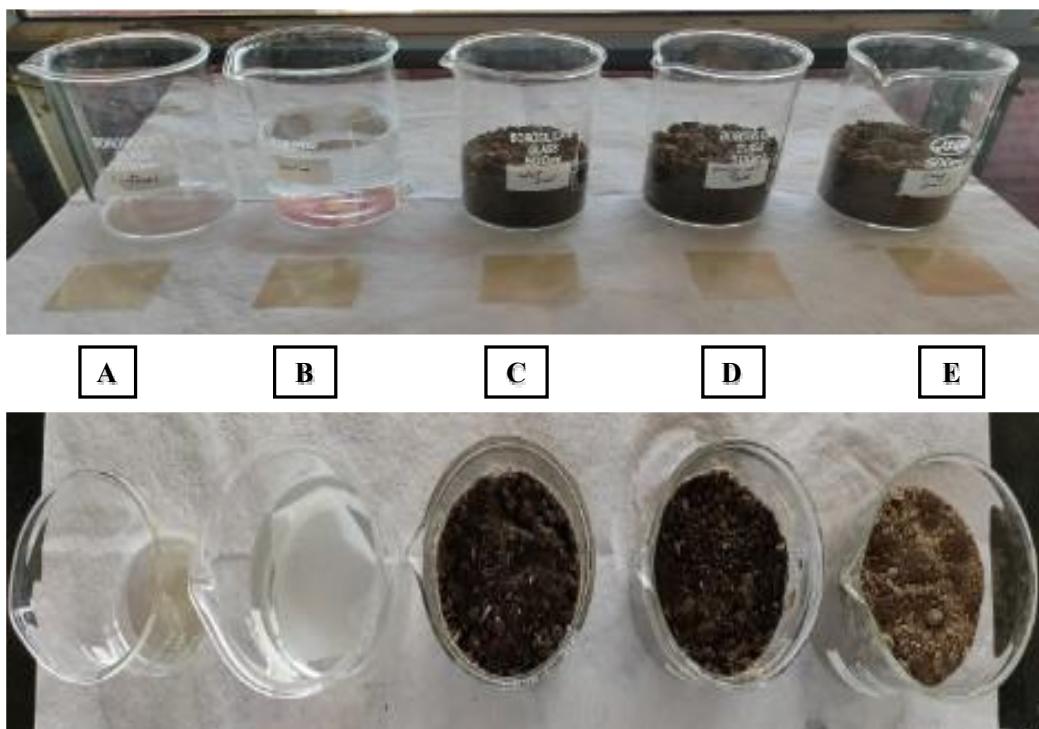


Figure 4.10: Sets of environmental condition. A is control, B is film in water sample, C is wet soil sample, D is daily wet soil sample and E is dry soil sample.

- Control

In control condition, film was leaved as it is in beaker to check the moisture absorbance from the air and to check the shelf life of the film at room temperature and it is found that film didn't retain moisture and it does not change its morphology and initial dry weight (260 mg) around one month which shows the film have good shelf life [73].



Figure 4.11: Biopolymeric film in control condition. A is 1st day film in an open beaker. B is 30th day film.

- Water

In this condition, the film was submerged in a beaker having tap water to check the degradation time of biopolymeric film. The biopolymeric film of initial weight 331 mg get partially soluble or partially degraded in 4 days. Due to the insoluble nature [47] of the keratin protein and hygroscopic nature [69] of gelatin the synthesized film only gets partially hydrolysed.

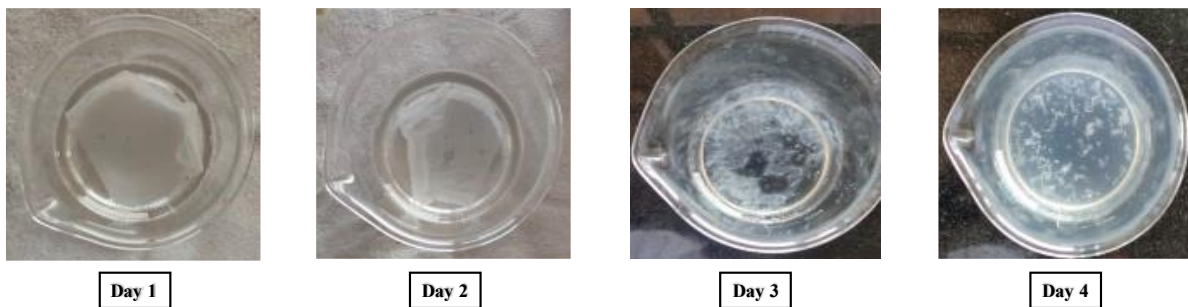


Figure 4.12: Biopolymeric Film in water from day 1 to day 4.

- Wet soil

In this condition, the biopolymeric film was put in wet soil sample in a beaker in order to check the biodegradability time, which was found that within 3 days the biopolymeric film sample having initial weight of 293 mg get degraded. Both keratin and gelatin are natural protein that's why this film get easily degraded [50] [79] in the wet soil sample in less time and also the gelatin is water and moisture sensitive so exposing to such condition make biodegradation of this biopolymeric film more favourable.



Figure 4.13: Biopolymeric film in wet soil sample. A is film before degradation at day 1. B is film after degradation at day 3.

- Daily wet soil

In this condition, the biopolymeric film was put in a beaker having soil sample which get daily watered in order to check the biodegradability time and it was found that within 2 days the biopolymeric film sample having initial weight of 340 mg get degraded. Both keratin and gelatin are natural protein that's why this film get easily degraded [50] [79] in less time and also the gelatin is water and moisture sensitive so exposing to such condition make biodegradation of this biopolymeric film more favourable. Daily watering the soil means giving extra moisture to the soil which results into faster degradation of the biopolymeric film.



Figure 4.14: Biopolymeric film in daily wet soil sample. A is film before degradation at day 1. B is film after degradation at day 2.

- Dry soil

In this condition, the biopolymeric film was put in a beaker having dry soil sample in order to check the biodegradability time. This condition take longer degradation time than other condition because the dry soil sample lacks the moisture and the degradation of the biopolymeric film was only depend on the soil rich enzymes and minerals. That's why the biopolymeric film sample having initial weight of 255 mg get degraded in 15 days. The lack of moisture and humidity makes the biodegradation rate of the biopolymeric film slow compare to others condition.



Figure 4.15: Biopolymeric film in dry soil sample. A is film before degradation at day 1. B is film after degradation at day 14.

Table 4.5: Biodegradability test of biopolymeric film under different environmental condition.

S.No.	Condition	Weight of the film (g)	Dimension of the film (cm)	Observation and Result
A	Control	0.260	5x5	Film does not change its morphology till one month
B	Water	0.331	5x5	Get partially soluble and partially degraded in 4 days
C	Wet soil	0.293	5x5	Degraded in 3 days
D	Daily wet soil	0.340	5x5	Degraded in 2 days
E	Dry soil	0.255	5x5	Degraded in 14 days

Results obtained in the biodegradability test proves that the synthesized keratin-gelatin blended biopolymeric film have biodegradable nature and can be easily degraded in natural environmental condition.

4.4.3 Mechanical properties

The testing of the synthesized keratin-gelatin biopolymeric film has been in progress.

CHAPTER-5

CONCLUSION

Bioplastic is need of the hour due to increasing plastic waste pollution which are causing many hazardous and unavoidable problems throughout the world. Due to green and biodegradable nature of bioplastics it can be a potential alternative of synthetic plastics which are fossil oil based and harms the environment as well. Chicken feather are rich in keratin protein which can be utilized as a natural biopolymer for the synthesis of bioplastic or biopolymeric film which has been reported in literature also. Chicken feather is considerably treated as waste and dumped in landfills which causes soil pollution. Utilization of these waste chicken feather after pre-treatment can solve two major problems i.e., waste management of chicken feathers and formation of cost-effective alternative of synthetic plastic which will be economical and sustainable. By applying alkaline and acidic treatment, chicken feathers can be efficiently hydrolyzed in order to make keratin protein rich solution or keratin powder.

There are various methods which was reported in literature earlier by different researchers that can be utilized and modified to create better keratin-based bioplastics. In various studies it is also showed that by using plasticizers like glycerol, ethylene glycol with keratin, bioplastic or biopolymeric film can be formed. This study was focused on production of keratin-based biopolymeric film by blending with other natural biopolymers like gelatin, cellulose and starch in order to enhance the properties of the film. So, that it can be utilized for various application like in soap packaging, food packaging or bio-packaging which have biomedical or industrial value.

Gelatin is a well-known protein which was extracted from collagen of animals having polymeric ability is blended with the dialyzed keratin solution to synthesized bioplastic film in this study. Gelatin is used as film forming agent in this study. The initial study of keratin-gelatin blended biopolymeric film formation in 0.5% glycerol (plasticizer) solution showed good results and in optimization experiments it was revealed that more than 1 % of gelatin and around 1.5 to 2% of keratin is required to synthesize an efficient biopolymeric film. The film formed was thin, transparent and durable. The solubility test revealed the hygroscopic and partially hydrophilic nature of the film and biodegradability test showed that the synthesized film can be easily biodegrade in natural environment in less time. The characterization of this synthesized keratin-gelatin blended biopolymeric film can reveal physical and mechanical properties which is still in progress. The characterization of the film can be done by using

different techniques like Fourier Transform Infrared (FTIR), X-ray Diffraction (XRD), Scanning Electron Microscope (SEM), Thermal Gravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC) etc.

In last it can be said that producing biopolymeric film or bioplastic by using keratin protein extracting from waste chicken feather can be a game changer in the biopolymer industry and it can be a potential and sustainable alternative of fossil oil-based plastic. This study is in budding phase which needs more research, knowledge and experiments for the production of bioplastic based on keratin.

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