

Review Article Reducing Meat Perishability through Pullulan Active Packaging

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The provision of safe products from the meat industry has been considered as the major source of protein for maintaining human health. Meat-borne outbreaks are mainly due to *Salmonella typhimurium* (*S. typhimurium*), *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), and *Clostridium perfringens* (*C. perfringens*), reducing the shelf life and consumer demands. A variety of vulnerable substances, including cholesterol oxidation products (COPs), are generated by the oxidation of meat induced by the microbial infestations. The use of certain biodegradable active packaging, including pullulan active packaging, is being focused by the meat industry due to their safety, stability, and negligible health risks. The potential of pullulan active packaging, incorporated with silver nanoparticles and essential oils, against *E. coli*, *S. typhimurium*, Mycoplasma, and other bacterial species is exclusive. Similarly, maintenance of organoleptic properties of meat with nominal oxidative rancidity and limited human health issues can be acquired by pullulan active packaging.

1. Introduction

The meat industry is facing several issues, and wastage of meat and meat products due to improper processing is the leading issue. According to an alarming report by the Food and Agriculture Organization (FAO) of the United Nations, approximately one-third or over 1.3 billion metric tons of all edible food products, including meat, is wasted annually due to poor practices in harvesting, storage, and transport [1]. Edible food items, especially meat, are also prone to chemical deterioration and microbial infestation causing the stunning threat to consumer health. These deteriorations represent huge economic losses to the food and meat industry every year. Due to the damaged quality of food items, the consumer confidence is being shaken. As a result, economic losses as well as litigation costs borne by food and meat industries are increasing day by day [2].

The losses may be greater in those areas and countries having less processing and sanitary control facilities. The

foodborne pathogens in these countries cause a regular health issue for the tourists and consumers in the export regions [3]. The broiler meat industry is facing huge wastage and losses due to improper processing and preservation. Every year, the spoilage of raw meat could be as high as 40% along with the food supply chain (production, retailing, and consumers) representing a remarkable loss [4]. Alternative packaging strategies, such as designing various pack sizes according to consumer demands and manipulative processing and packaging to maintain food quality and shelf life, have been proposed to reduce these food losses [5]. This reduction in food wastage would enable the nations for sustainable food production and enhance the market development. Globally, more emphasis is done on food safety and it is one of the major objectives of the recent food legislation. The microbiological risks of food products, especially broiler meat, are the major sources of foodborne outbreaks which resulted in a high causalities rate of 13% around the world [6]. In addition to microbiological safety,

the colour and lipid stability of meat are the salient parameters of quality influenced by consumer acceptance [7, 8]. For extending the shelf life of meat and meat products, antimicrobially active, commercially based, economical, and smart preservation solution is "nanotechnology" [9]. Moreover, nanotechnology enables meat preservation with certain biological and chemical procedures by maintaining the compositional integrity, moisture, and gaseous exchange from the meat surface [10]. This advanced technology has a prominent impact on production, storage, processing, transportation, and safety of meat and meat products [11].

For the preservation of meat and meat products, many synthetic and natural antimicrobials/antioxidants have, continuously, been used to reduce meat spoilage with easier commercialization of meat [12]. These products were used either onto the meat surface, in the diet of animals, or along with edible films and coatings (EFCs) mostly referred as "active packaging" [12]. EFCs directly produced or obtained by biomass (polysaccharides and protein), synthesized chemically (i.e., polylactic acid), or obtained from microorganisms (i.e., pullulan) are most preferably utilized as active or biodegradable packaging [13]. The efficacy of EFCs has been investigated by many researchers in previous decades focusing on improvement of their effectiveness when incorporated with natural or metal nanoparticles [13, 14].

Pullulan is one of the best used biodegradable polysaccharides capable of forming better, colourless, and tasteless edible active packaging with better oxygen and gas barrier capacities [15]. The incorporation of various antimicrobial agents (including organic acids, essential oils, plant extracts, proteins, and metal nanoparticles) into "pullulan active packaging" can reduce the major microorganism species load in meat products [14].

Based on these facts, this review is aiming on the application of pullulan active packaging for efficient and prolonged meat preservation. In our review, we have provided comprehensive details regarding meat-borne outbreaks, meat oxidation, cholesterol oxidation products (COPs), use of active packaging, and pullulan active packaging for meat preservation.

1.1. Meat-Borne Outbreaks. Meat-borne outbreaks are mostly due to S. typhimurium, S. aureus, E. coli, and C. perfringens [6, 16]. The red meat-related outbreaks are mainly due to the infestation by Salmonella species, Listeria species, Staphylococcus species, and Clostridium species, and most of the outbreaks were due to S. typhimurium [5]. In the case of broiler meat-related outbreaks, most of the food poisoning incidences were due to S. typhimurium, C. perfringens, and Campylobacter species [5]. Most of the countries are prone to the attack of the bacterial strains in food items due to favourable environmental conditions [17-19]. This problem may be induced by lack of basic principles for food processing, unhygienic food packaging, preservation, and inappropriate microbiological safety measures during the processing of food items especially the meat [20, 21].

As preventive measures, the processing, preservation, packaging, storage, transportation, and distribution of meat and meat products should be in a hygienic environment [22]. When meat and poultry sectors do not compromise to compel minimum criteria to ensure the safety and quality of their products, the foodborne outbreaks will not be subsidized [20]. Meat industry must monitor the microbiological load and other biological hazards, causing potential hazards for consumer health, during the production and processing of food items [23]. The meat-related outbreaks occur during cutting and processing, cooking, packaging, transportation, storage, and preservation [24]. These meat-related outbreaks are not only causing serious threats to human beings in the last decade but also deteriorate meat quality in the form of "lipid and protein oxidation" [25].

Implementation of regulatory requirements, good manufacturing, and hygiene practices may be considered as a good methodology to achieve food safety all over the globe [23].

1.2. Meat Oxidation. The pathogenic species of bacteria can deteriorate the quality and shelf life of meat and meat products. Meat deterioration may be experienced as discoloration, off flavour, reduced shelf life, toxic compound formation in meat and meat products, and drip losses of the nutrients [3, 25, 26]. These negative changes in meat and meat products are, mainly, due to oxidation of lipids and protein. This process is affected by the temperature and duration of their storage [27]. The amount of COPs in meat is a reckonable parameter to detect lipid oxidation or "oxidative rancidity" [28]. Similarly, the adaptability of certain microbial strains against new weather and environmental stress is triggered by oxidative rancidity leading towards the production of highly vulnerable substances [29]. The customer demands may be declined by this pessimistic transformation in meat and meat products influenced by environment or storage conditions. These qualitative changes mainly happen due to "oxidation" of lipids resulting in the formation of COPs or protein oxidation [30]. When the packed fresh meat, in a transparent packaging material, is exposed to sunlight/oxygen or improperly stored, the oxidation is initiated, leading towards the reduction in the organoleptic or sensory properties with a higher rejection by the valued customers [31].

Protein oxidation is mainly induced by the bacterial species or due to the oxidative stress [32]. Covalent modification of protein molecules during the oxidation process not only harms the organoleptic properties of the meat and meat products but also influences human health [32].

COPs are formed when the animal-derived food products are heated, cooked dehydrated, stored, and exposed to radiations [33], reducing their demands especially in the persons having high blood cholesterol. Various food products reflect the changing amount of COP formation during processing and storage (Table 1) due to the lipid contents [30].

COP generation is, mainly, conjugated with the amount of fatty acids (unsaturated) and cholesterol in meat,

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Food products (stored/processed)	Lipid percentages (%)	COPs (g/ 100 g lipids)
Raw beef	13.8	0.50
Cooked beef	10.6-12.2	0.40-0.50
Whole egg powder	44	0.80-1.50
Whole milk powder	22.7	0.30-0.40
Grated cheese (Grana type)	18.4-28.4	0.30-0.40
Snacks prepared with eggs	12.7-30.9	0.4-2.60
Biscuits prepared with eggs	3.7-27.6	0.20-3.70

radiations (ultraviolet and gamma), moisture contents, and packaging materials [33, 34]. Addition of naturally available phenolic compounds (i.e., tannic acid and catechin) to the food products and raw meat may be beneficial to reduce the microbial growth and lipid oxidation [34]. The amount of COPs will be increased when the raw meat is packed and stored while they may be reduced when the processing/ packaging of meat is carried out after cooking [35]. Similarly, vacuum packaging and a low amount of fatty acids in meat can also be considered as "tools" to reduce the COPs in the meat during processing [35]. The maintenance of the quality of meat is still a question mark for the food scientists. These vital issues of the commercial meat industry require appropriate attention by the researchers and government agencies to decrease the losses in near future.

2. Meat Active Packaging

The shelf life of meat has been prolonged by the use of synthetic antioxidants including butylated hydroxyl-toluene (BHT), butylated hydroxyl-anisole (BHA), and propyl gallate (PG) to reduce "oxidative rancidity" of fats [35]. However, human health safety is the major concern of the researchers regarding the extensive usage of BHT, BHA, and PG, switching the direction towards natural products [33, 35]. Deterioration of meat and meat products is also due to microbial infestation by native microflora in the gut of the animals, through environment, improper handling, transportation, and preservation methods [11]. To overcome these problems, antimicrobials are added to meat and meat products. The preservation of meat by using antimicrobial or antioxidant additives is considered hazardous for human health, and the development of green preservatives is highly required [12]. A study by Nithya and Mohankumar [36] showed that the pathogenic strains of bacteria, especially present in poultry meat, exhibit a maximum "multiple antibiotic resistance index" (MAR) especially S. typhimurium, E. coli, S. aureus, Vibrio cholera (V. cholera), Campylobacter jejuni (C. jejuni), and Listeria monocytogenes (L. monocytogenes). This issue is alarming as the ingestion of these pathogenic bacterial strains can cause huge outbreaks of foodborne problems due to their antibiotic resistance [37]. Moreover, physiochemical changes are continuously induced by these pathogens which may reduce the demands for meat and meat products [37]. EFCs are the best suited and green alternatives to maintain the quality and safety of meat products and to reduce microbial load [14]. The standardized procedures for the synthesis of green

packaging products according to industrial requirements and incorporation with "green" antioxidants/antimicrobials are the major challenges to improve meat preservation [14].

In the recent few decades, food scientists have been working on green synthesized preservatives to enhance the quality and shelf life of meat (both fresh and frozen). Ecofriendly, safe, and biodegradable food and meat preservation may help to reduce the risk to the entire ecosystem caused by conventional and synthetic preservatives [38]. This practice may increase the consumer demands towards fresh food materials with extended shelf life and quality ensuring maximum distribution channels all over the globe [37, 38]. Additionally, the consumer acceptances pertaining to the "sensory characteristics" with better health safety and economical aspects in usage are the major objectives for the formation of such green synthesized packaging materials [38]. The meat-based items provide excellent media for bacterial growth which may modify the overall organoleptic, sensory, and chemical parameters, resulting in the decrease of shelf life and higher foodborne illnesses as mentioned by Kerry [39]. Nanotechnology can be a simple, green, and economical solution to lessen these issues. It is assumed that if nanotechnology paces at its current rate in the meat industry, the global hunger can be defeated in a very shorter period with an ample supply of processed meat [40-44]. This advanced technology has a prominent impact on the production, storage, processing, transportation, and safety of poultry products [11, 45-47].

The naturally available/green synthesized products can be used to preserve the various food items especially the meat (Table 2) due to their biodegradable qualities [16, 17].

Plant extracts and essential oils cannot be used without any active packaging for the preservation of food due to their variable characteristics and bioavailability. Active edible packaging incorporated with plant extracts and essential oils is a better choice for the long-term preservation of meat and meat products [12, 48–50].

The meat industry requires wide use of natural or green synthesized products to improve the quality of meat and meat products experienced by the consumers [11, 50, 51]. By this practice, excessive usage of synthetic meat preservatives can be reduced to increase the organoleptic and physicochemical properties of meat and meat products [14, 52]. Antimicrobial/antioxidant efficacy of the active substances in active packaging is, mainly, dependent on their uptake pathways, size, shape, composition, and surface modifications [53–62]. Moreover, the cellular and subcellular distribution of active compounds, from active packaging, to the

TABLE	2:	Natural	green	synthesized	active	packaging	[16, 1	[7].

Natural/green synthesized packaging products	Type of food	Active ingredient
Chitosan	Chicken breasts, cheese, and tilapia fillets	Organic acids
Starch/glycerol	Chicken breast meat, tilapia fillets, and cheese	Organic acids
Chitosan	Ground beef	Grapefruit extracts
Horseradish extracts	Beef	_
Clove, citronella, and cyprus	Food packaging in films	Clover oil
Seaweed extracts	Fruits, vegetables, and meat	—
Alginates	—	Polysaccharides and glycoprotein
Pullulan	Meat, eggs, and fruits	Polysaccharides

TABLE 3: Antimicrobial active packaging.

Active ingredi	ents					
Edible films	Incorporated materials		Active against bacterial species	Techniques developed	References	
and coatings Nanoparticles		Others				
Chitosan	Silver	Ciprofloxacin	P. aeruginosa	Antimicrobial loaded films	[70]	
Chitosan	Zinc oxide	Carboxymethyl cellulose	S. aureus P. aeruginosa, B. cereus	Bionanocomposite films		
_	—	Essential oils	S. typhimurium, L. monocytogenes, E. coli	Low-density polyethylene packaging (LDPE)		
_	—	Phenolic acid—gallic acid, benzoic acid, and flavonoids	L. monocytogenes, C. jejuni	Flexible bioactive packaging		
Whey proteins	—	Essential oils	E. coli, L. monocytogenes, S. aureus, S. enteritidis E. coli, L. monocytogenes,	Edible films from whey proteins		
Chitosan	—	Garlic oil	S. aureus, B. cereus, S. typhimurium	Edible films from chitosan		
Milk protein	—	Organic acids	E. coli and Pseudomonas spp.	Milk protein-based edible films		
_	—	Chitosonium acetate	L. monocytogenes, S. aureus, Salmonella spp.	Antimicrobial packaging		
Cellulose	Silver	—	S. aureus, E. coli	Active food packaging	[71]	
Chitosan	Silver	Mandarin essential oil	L. innocua	Modified coating	[72]	
Pullulan	Silver	—	B. subtilis, S. aureus, S. marcescens	Pullulan-silver nanopackaging	[73]	
Alginate	Zinc oxide	Glycerol	S. aureus, S. typhimurium	Active packaging	[74]	
Alginate	_	Cinnamon bark oil and soybean oil	E. coli, L. monocytogenes, and Salmonella enterica	Edible film packaging	[75]	
Soybean	Zinc oxide	· _	S. aureus and E. coli	Active packaging	[76]	

food surface also governs their toxicity risks [61–67]. During the last few years, the development of suitable active packaging, with the lowest toxicity, gained the highest demands by meat and food industry with a better antimicrobial impact and customer safety [14, 67, 68]. Prolonged and microbial free meat preservation requires the application of better technology, such as EFCs, to ensure the quality of processed meat [52, 69]; Table 3. It will not only generate more revenue by the meat industry but also maintain customer demand and health.

For the long-term preservation of meat and meat products, emulsifiers and surface-active agents are also used as gas and moisture barriers in the form of "meat coatings" [14]. Pure lipids combined with hydrocolloids (such as protein, starch, cellulose, and their derivatives) may act as a component system able to be applied as "meat coatings" [69]. In fresh and processed meats, lipid incorporation into EFCs makes an excellent water barrier to improve the hydrophobicity, cohesiveness, and flexibility, leading to prolongation of freshness, colour, aroma, tenderness, and microbiological stability. Despite various advantages, protein films may be prone to proteolytic degradation in the presence of enzymes in meat products, or allergenic protein fractions can cause adverse effects on the susceptible people [77].

Polysaccharide-based films and coatings enhance the shelf life of meat and meat products due to the prevention of oxidative rancidity, dehydration, and surface browning [14]. These films dissolve and become integrated into the wrapped meat products exposed to smoke and steam. This parameter reduces the moisture loss from the stored meat products and improves the structure and texture, resulting in higher yields [69]. Moreover, polysaccharidebased films and coatings provide crispiness, hardness, viscosity, compactness, and gel-forming quality to the films.

3. Pullulan Active Packaging for Meat Preservation

Pullulan is an extracellular polysaccharide produced by a fungal species *Aureobasidium pullulans* [78] which is chemically constructed by repeated maltofructose units (Figure 1). This bioavailable polysaccharide is not exploited yet as "natural preservative and packaging material" except its application against rancidity [79].

Pullulan edible films are considered as more homogeneous and translucent with improved thermal stability and tensile strength when compared with other polysaccharidebased edible films, i.e., chitosan or seaweed edible films [80]. Further, this linear polymer exhibits the viscoelastic behaviour upon the drying and provides the entangled network of the fibers as compared to other bioavailable polymers, presenting less evaporation of water at complete drying [81, 82]. Studies conducted by Diab et al. [38] and Cheng et al. [78] revealed that active packaging obtained from pullulan showed colourless and tasteless texture that was resistant to oil and exhibited very low oxygen permeability with better barrier characteristics as compared to other edible films.

Pullulan active packaging, containing the sweet basil extract (SBE), reduces the chances of weight loss and colour changes in the stored food [82]. Chitosan active packaging (as compared to pullulan) may lead towards the intermolecular hydrogen bond formation instead of intramolecular hydrogen bonding, causing drastic changes in XRD (X-ray diffraction) measurements [82]. These results indicate that pullulan active packaging releases the silver ions in better quantity as compared to chitosan films [82]. Similarly, pullulan active packaging is considered as one of the emerging preservation technologies, to ensure the microbial safety and meat preservation, rarely influenced by external factors [83]. When the food materials are preserved with these packaging, the delicacy and originality of food taste are secured and preserved [14]. A study conducted by Karolina et al. [15] proved that pullulan active packaging preserves the quality of all food items (including fruits and meat) with safety against physical and mechanical damages. Incorporation of nanoparticles in pullulan film to control the foodborne pathogens of meat and meat products provided a new chapter in food and meat processing [84, 85].

3.1. Mechanism of Action. The incorporation of nanoparticles into pullulan provides tremendous antimicrobial activity against *E. coli* and *L. monocytogenes* by slow release of inorganic nanoparticles to targeted bacterial cells [86–88]. The delivery of specific "antimicrobial" substance directly to the surface of meat can reduce the population of various bacterial strains [89]. Antimicrobial mechanism of any antimicrobial substance incorporated into pullulan active packaging includes the following:

(a) Prolongation of the lag phase of growth of bacteria/ fungi [55]

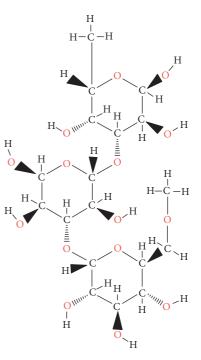


FIGURE 1: Pullulan chemical structure with repeated maltofructose units.

- (b) Cell wall damage [62]
- (c) Formation of holes and gaps in the outer membrane of bacterial/fungal cells [54]
- (d) Destruction of the outer and inner membrane due to the penetration of metabolites into the bacterial/ fungal cells resulting in the death of the target organism [54]
- (e) Loss of integrity of cell membrane and cytoplasmic membrane which increase bacterial/fungal cell permeability [57, 58]

The antimicrobials or the active substances include silver nanoparticles, gold nanoparticles, TiO₂ nanoparticles, essential oils, and certain bacteriocins [90]. The bacteriocins are the peptides (proteins) synthesized by the ribosome of one strain of bacteria with an antimicrobial capacity against their allied strains [91, 92]. The delivery of nanoparticles, essential oils, and bacteriocins, incorporated in pullulan edible films, direct to the surface of meat can maintain its quality and prolongs the storage duration [91]. This is, directly, related to the fact that pullulan can adhere to the moist surface of meat and releases the "active agents" (nanoparticles, essential oils, and bacteriocins) right into the surface slowly [86]. It can surround nanoparticles in the form of a thin layer as well resulting in the reduction of the number of harmful bacterial strains [46].

Incorporation of nanoparticles/active substances from different origins, into pullulan, to formulate "active packaging" has been reported by many researchers in the last two decades, as a green preservative, to prolong the shelf life and quality of meat [85, 88, 91] (Table 4).

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Effective against bacterial strains	Type of food items	References
S. aureus, L. monocytogenes, S. typhimurium, and E. coli O157:H7	Poultry meat products	[84]
S. aureus, L. monocytogenes, E. coli O157:H7, and S. typhimurium	Turkey meat	[86]
S. aureus and L. monocytogenes.	Turkey meat	[86]
L. monocytogenes and S. aureus	Turkey deli meat	[86]
S. aureus, L. monocytogenes, and E. coli O157:H7	Ham deli meat and raw beef	[87]
S. typhimurium and S. enteritidis	Turkey breast	[88]
	S. aureus, L. monocytogenes, S. typhimurium, and E. coli O157:H7 S. aureus, L. monocytogenes, E. coli O157:H7, and S. typhimurium S. aureus and L. monocytogenes. L. monocytogenes and S. aureus S. aureus, L. monocytogenes, and E. coli O157:H7	S. aureus, L. monocytogenes, S. typhimurium, and E. coli O157:H7 Poultry meat products S. aureus, L. monocytogenes, E. coli O157:H7, and S. typhimurium Turkey meat S. aureus and L. monocytogenes. L. monocytogenes and S. aureus Turkey meat S. aureus, L. monocytogenes, and E. coli O157:H7 Ham deli meat and raw beef

Similarly, antimicrobial characteristics of silver nanoparticles (AgNPs) incorporated into active packaging, against various dangerous species of bacteria, cannot be ignored [79]. AgNPs synthesized from curcumin, pullulan, cellulose, and collagen sources can act as a strong antibacterial and antifungal agent [46, 91, 92]. For the customer health protection and product quality maintenance, the green synthesized AgNPs from pullulan (as stabilizing/reducing agent) can show tremendous results [14]. The use of "pullulan active packaging," incorporated with AgNPs is increasing day by day in the meat industry as a "green" preservative [85]. However, various factors may also affect the antimicrobial activity of "pullulan active packaging," incorporated with AgNPs including size, shape, and crystallographic structure of AgNPs [92]. Ganduri et al. [73] proved that the AgNPs, synthesized from the increasing amount of pullulan concentrations, showed an intense antimicrobial capacity for meat preservation [69]. Moreover, pullulan-mediated AgNPs can be utilized as an "active substance" for the preservation of meat and meat products, but the size, hydrophilic-hydrophobic character of pullulan-mediated AgNPs, and their concentration are the major factors affecting their efficacy [46, 93]. For the preservation of meat and meat products, the incorporation of silver nanoparticles into pullulan films to formulate "active packaging" is ever-increasing [85]. The conjugation of antimicrobial abilities of pullulan and AgNPs can improve the shelf life and quality of meat products [46, 92]. Khalaf et al. [86] reported that pullulan active films, containing silver nanoparticles, surprisingly inactivated the lethal impacts of L. monocytogenes and S. aureus causing a reduction in the spoilage of meat and meat products. The mentioned study was performed on turkey meat showed the antimicrobial activity of pullulan-incorporated silver nanoparticles for the safe processing of white meat. The stability of pullulan active packaging, incorporated with AgNPs, at a wide range of storage temperature, i.e., 4°C to 25°C, showed a novel route for safe, healthier, and biodegradable meat packaging [86, 94-97].

The superior biocompatibility, nonimmunogenic, nonmutagenic, noncarcinogenic, and easily degradable properties of pullulan-based active packaging can be considered as a better choice to improve the quality and shelf life of meat in preservation studies [98–100].

These advancements for meat processing can further reduce the microbial contamination in broiler meat with improved quality [101, 102].

4. Conclusion

The use of synthetic antioxidants (butylated hydroxyl-toluene, butylated hydroxyl-anisole, and propyl gallate) and antimicrobials, to overcome meat oxidation and perishability, is imposing serious health issues in human beings. The biodegradable pullulan active packaging, incorporated with any active substance (i.e., AgNPs and essential oils) may be adopted as the alternative technology due to their superior antimicrobial capacity against S. typhimurium, E. coli, S. aureus, C. perfringens, V. cholera, C. jejuni, S. enteritidis, and L. monocytogenes. Due to the effect of pullulan active packaging on microbial population reduction, the oxidative and compositional stability of meat is remained unchanged, resulting in higher customer safety. Furthermore, antimicrobial activity of AgNPs released from the active packaging is one of the complex phenomena influenced by their better composition, appropriate size, and shape. The green and reliable pullulan active meat packaging can not only reduce the chances of meat perishability but also lower down the oxidative rancidity, generation of COPs, and compositional losses.

Data Availability

No experimental data was utilized in this manuscript. The literature and referred journal research papers were considered.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Sazili AQ, Kumari S, and Khan MJ conceptualized the study; Khan MJ was involved in data curation and responsible for software development and wrote the original draft; Khan MJ, Sazili AQ, and Kumari S investigated the study; Khan MJ, Kumari S, Selamat J, Shameli K, and Sazili AQ wrote, reviewed, and edited the manuscript.

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