# 3 Materials from Natural Resources for the Application of Bone Tissue Engineering

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**Abstract**

Natural materials have distinctive qualities like the ability to communicate with the biological environment, especially during the regeneration process. In this perspective, natural materials exhibit significant bioactive attributes because they were synthesized from nature to carry out a particular task. Natural materials can be extracted from various sources, but their chemistry and bioactivity may differ noticeably depending on the organism under consideration and the extraction technique employed. Composite scaffolds for bone tissue engineering have been developed from natural origin. The balancing act of mechanical strength, porosity, and growth factors in bone scaffolds is still challenging. This chapter brings light to the composition of bone and the importance of bone and its tissue engineering. Further, this chapter will discuss the materials, e.g., calcium phosphorous, bioglass, collagen, chitosan hyaluronic acid, cellulose, starch, and alginate, that can be used as efficient materials for bone tissue engineering.

**Keywords**

Calcium phosphorous · Bioglass · Collagen · Chitosan · Hyaluronic acid · Cellulose

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## 3.1 Introduction

Bone is vascularized tissue composed of outer hard, compact, and inner soft cancellous tissue. Collagen makes up the majority of bone's organic component (type-I), which supports the bone structure. The inorganic phases are mainly calcium phosphate. The four kinds of bone cells are osteoblast, osteoclast, osteocytes, and osteoprogenitor cells. The repair of bone defects is facilitated by osteoclast and osteoblast cell activity by balancing bone resorption and bone formation. Tissue engineering is an emerging approach to developing a new methodology for bone regeneration (Guo et al. 2021).

Mainly studied synthetic scaffold materials are ceramics, polymers, and their composites. The synthetic materials showed many disadvantages, like unsatisfactory cell growth and inadequate cell adhesion ability due to the unavailability of functional groups on their surface. These shortcomings can be compensated by the use of natural-based biomaterials. There are many benefits of the use of natural biomaterials, such as their biomimetic properties, ecological safety, and huge natural resource with minimal processing and synthesis cost. Also, natural biomaterials show good biodegradable and biocompatible properties (Barua et al. 2018). Two types of natural materials are utilized in tissue engineering applications: biopolymers and bioceramics.

## 3.2 Bone Tissue Engineering

The production of biological substitutes that rebuild, retain, and enhance the activity of organs or tissue is the aim of tissue engineering, which combines engineering and life science. Since the second most transplanted tissue is bone, it has garnered much interest among the many tissue types. To enable their tasks, the organic and inorganic components of bone interact and maintain physiochemical balance in a complex tissue. To resemble bone, a scaffold for bone tissue creation should have characteristics similar to those of bone extracellular matrix. Bone structure, bone mechanics, and bone tissue development are studied in bone tissue engineering, in other words, understanding the biology of bone and its ability to regenerate or repair bone.

Long bones are developed through endochondral ossification, which results in the mineralization of cartilage. Contrarily, flat bones are created through an intramembranous ossification process where mesenchymal stem cells develop osteoblasts that build bone. Osteoblasts release alkaline phosphatase, which encourages the retention of calcium and phosphate ions along with collagen fibers and unmineralized collagen (osteoid) (Zeng et al. 2017). After maturing into osteocytes, which monitor the homeostasis of bone, detect regional mechanical atmosphere, and react through balancing bone formation and resorption, osteoblasts are finally developed which are surrounded by a mineralized bone matrix (Yang et al. 2014). Bone growth and fracture healing need bone remodeling, the technique by which functional modification of the bone tissue framework takes place in

**Table 3.1** Design conditions of scaffolds in bone tissue engineering (Thavornyutikarn et al. 2014)

| Design criteria | Conditions |
|---|---|
| Porosity | Without compromising mechanical properties, as much as possible |
| Pore architecture | Interconnected pores with a diameter of 200–400 μm |
| Degradation behavior | The degradation time must be adaptable according to the requirements |
| Biocompatibility | No persistent inflammation |
| Sterilizability | Without affecting the material's characteristics |

response to mechanical stress variations. Osteoclasts are multinucleated cells that can develop from progenitor monocytes into macrophages (immune cells) and perform bone resorption. Macrophages take part in the inflammatory response that encourages MSCs to migrate to the fracture site and consequently improves the repair of the fracture (Pajarinen et al. 2019).

Additionally, macrophages play important roles in bone formation and homeostasis, and MSCs demonstrate immunomodulatory functions. Bioceramics and polymers are potential candidates for biomaterials that resemble the organic (collagen) and inorganic (hydroxyapatite) components of the extracellular matrix (ECM) of bone. Before mineralization, successive cross-linking of collagen fibrils occurs during ECM production, gradually improving the mechanical behavior of ECM (Dhand et al. 2016). Crystalline bioactive ceramics and amorphous bioactive glasses are both examples of bioceramics. Polymers can be created artificially or naturally, like collagen. Due to their higher mechanical performance, biodegradable metals and nanomaterials made of carbon are also employed. To stimulate developmental signaling cascades biochemically, mechanically, or piezoelectrically, biomaterials can be created for bone tissue engineering. The nanotopography of an immunomodulatory substance can simulate bone nanointerfaces by steering macrophages toward bone-stimulating activity (Damaraju et al. 2017). Bone tissue engineering aims to create biomaterials that can take the place of allografts and autografts. The material should help in bone remodeling when implanted in the defect area (Koons et al. 2020). The basic building blocks of bone tissue engineering are progenitor cells (that express tissue matrix), scaffolds (provisionally support bone formation), and growth factors (stimulate osteoblast regeneration). Table 3.1 summarizes the particular standards for the optimum scaffold in bone tissue engineering.

## 3.3 Bone Regeneration

In the healing response after an injury or during skeletal growth, bone can naturally regenerate. Bone regeneration is a series of organized biological events, i.e., bone induction and conduction, that involve many cells and intracellular and extracellular molecular signaling pathways. Fracture repair, including intramembranous and endochondral ossification, imitates the embryonic skeletogenesis process in its normal state.

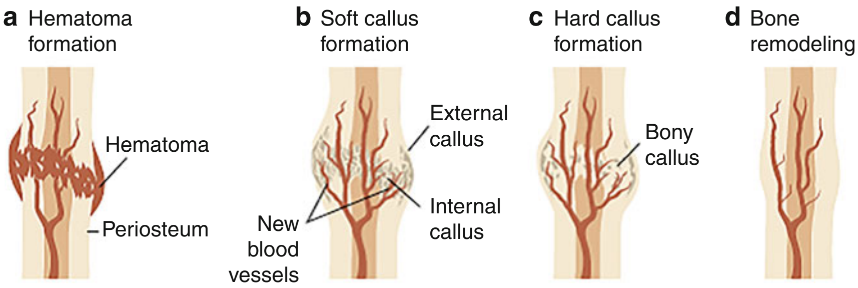


**Fig. 3.1** The stages of fracture repair (Carano and Filvaroff 2003). (Reproduced from open access publication)

Several elements work together in bone formation technique: (1) a variety of cells types like osteoclasts, MSCs, etc.; (2) biomaterial (scaffold); (3) effects of growth factors, vitamins, cytokines, hormones, ions, etc.; and (4) several impulses (mechanical). Figure 3.1 depicts the four overlapping phases that the callus formation process uses to repair fractures. After musculoskeletal system damage, blood vessel disruption causes the coagulation cascade to be activated, which then results in the development of hematoma around the fracture site (Fig. 3.1a). In line with the hematoma's angiogenic activity, removal of the hematoma considerably reduces healing, whereas transplantation of the hematoma results in new bone formation. In response to the attraction of stem cells, inflammatory cells, and fibroblasts to location, new blood vessels are formed from already-existing vessels, which is known as angiogenesis. The inflammatory reaction is accompanied by discomfort, warmness, redness, and the release of many cytokines and growth factors that are crucial for repair. At the extremities of the bones, initially, granulation tissue develops before progressively giving way to fibrocartilage in a manner that appears to be tied to the vascular pattern. While this is happening, an external callus is being formed on the periosteum through direct bone production, also known as intramembranous ossification (Fig. 3.1b). After that, the rigid callus of braided bone is formed from the internal callus after it has calcified with calcium hydroxyapatite (Fig. 3.1c). The fracture callus is replaced by secondary lamellar bone during the remodeling phase, which also sees the callus' size reduced to that of the preexisting bone in the damaged area and the vascular supply return to normal (Fig. 3.1d) (Carano and Filvaroff 2003).

### 3.3.1 Calcium Phosphate

Large bone defects, such as those caused by complex bone fractures and incurable bone diseases, pose particular difficulties for bone regeneration. It is essential for healing that implanted materials fill the bone deficiencies. Grafting with autologous bone is the most efficient approach to repairing bone defects (Park et al. 2022).

Bioceramics are a kind of biomaterials for biomedical applications. Because of their superior biocompatibility, mechanical properties, and other attributes, these types of biomaterials are used in bone tissue engineering. Calcium phosphates are widely used in orthopedic implants, which are readily available in the human bone and tooth. Calcium phosphates are bioactive, biocompatible, and osteoconductive (Mohd Pu'ad et al. 2019).

Due to its prevalence and significance among calcium compounds, hydroxyapatite is a mineral phase that is naturally present in hard tissues. The rigidity of bone, dentin, and enamel is a result of hydroxyapatite's role as reinforcement in hard tissues. HAp crystals are present in collagen as needlelike structures that are aligned in the direction of the fibers in cortical (compact) bone. However, in reality, it has a complex chemical makeup that is nonstoichiometric as well as calcium-deficient (Ca:P molar ratio 1.67) as a result of vacancies and foreign cations and anions that are absorbed from the nearby body fluids at the time of bone metabolism, including sodium, zinc, magnesium, iron, and carbonate (Begam et al. 2017). Currently, HAp has been investigated for applications in different areas like drug delivery, collagen stimulation, skin regeneration, sun protection, water purification, wastewater treatment, and so on (Tan et al. 2020; Steffens et al. 2015; Ibrahim et al. 2020; Ideia et al. 2021). As a result, numerous techniques for synthesizing HAp with specific properties have been explored. These can be divided into three categories: dry methods (solid-state and mechanochemical), wet-chemical methods (sol-gel method, chemical-precipitation method, hydrothermal reaction, hydrolysis method, polymer-assisted route, etc.), and high-temperature methods (combustion method and pyrolysis route).

The two types of carbonate HAp that mimic biological apatite are A-type and B-type carbonate HAp with hydroxyl ($OH^-$) and phosphate ($PO_4^{3-}$) group replacements, respectively. The structural, optical, and morphological properties of HAp are greatly influenced by the presence of ions (Vinoth Kumar et al. 2021). Natural HAp is typically taken from organic materials or waste materials like plants, algae, cockles, clams, fish scales, and mammalian bones (such as those from cattle, camels, and horses). It can also be extracted from mineral materials (e.g., limestone). It has been demonstrated that this ratio works best to encourage bone repair. Because natural HAp is not stoichiometric, it either has a calcium or phosphorus deficiency (Ratner et al. 2012). The most frequent vacancies in HAp are in the calcium sites, which are filled by cations including $Na^+$, $Mg^{2+}$, and $Al^{3+}$ (Ratner et al. 2012). The trace elements speed up the process of bone production and are crucial for bone repair.

In general, HAp crystallites can exist in a variety of shapes, including needles, rods, spheres, and others. Several variables, including extraction method, calcination temperature, and bone type, affect the characteristics, efficacy, phase purity, and particle size of HAp derived from a biological source (bone). Usually, the cortical region of the femoral bones is used to prepare scaffolds. To eradicate dust and proteins from the bone surface, bones are washed thoroughly, boiled in distilled water, and then treated with hypochlorite or NaOH. Following this initial cleaning and drying, bones are crushed or subjected to ball milling, impacting the end product's morphology and particle size. To eliminate the organic components from the bone and produce HAp, bones are treated in a furnace (600 and 1400 °C) during the calcination process. To get rid of the organic material and improve HAp's crystallinity while preventing thermal degradation of the finished product, a carefully chosen calcination regime is used. The germs that could spread diseases from the cattle to the patient are also eliminated by calcination treatment (Akram and Ahmed 2014).

The calcium carbonate content in cockle, clam, and eggshells is high, which can be transformed into HAp. Calcium can be obtained from eggshells, which have a calcium content of 94% $CaCO_3$. Typically, the shells were cleaned by first washing them in distilled water to get rid of sand and grime and then cleaning them again with bleach. CaO is obtained by calcining shells which will be used, along with surplus $CaCO_3$, for additional chemical processing to produce high-purity HAp (Mustaffa et al. 2015) (Table 3.2).

Usually, the form and sizes of the HAp produced from various sources differ. The extraction technique had an impact on the properties of the HAp that was produced. In contrast to chemical precipitation, heat methods (calcination) produce crystalline HAp. Some resources, like seashells, algae, etc., are abundant in $CaCO_3$ and are processed to prepare phase pure HAp. Therefore, a variety of techniques have been utilized to prepare phase pure HAp identical to the minerals found in human bone. Understanding HAp's characteristics, such as its phase purity and thermal stability, is crucial given the growing demand for it in biomedical applications, especially when isolated from natural sources. Furthermore, using natural resources to extract HAp ensures sustainability because they enable waste materials to be converted into value-added commodities by recovering nutrients from them.

### 3.3.2 Bioglass

Bioglass, also known as bioactive glass (BG), is widely used for clinical therapeutic purposes, notably in the orthopedic profession. The prerequisite for BG to heal bone fractures has increased as the human population has grown (Jones 2013). Since the landmark study by Hench et al. in 1972, BGs have attracted great interest for medical applications because of their high osteoconductive, bioactive, and biodegradable features (Rizwan et al. 2017). The implanted BGs have the ability to establish bond between implant materials and host tissues by creating an appetite layer on their surface that is comparable to bone minerals and can integrate with the host bone (Ibrahim et al. 2017). When new tissue is growing, bioglass releases physiologically active ions that encourage osteogenesis and induce vascular ingrowth (Gorustovich et al. 2009).

For the melt quench technique of 45S5 synthesis, silica, phosphorus pentoxide, calcium carbonate, and sodium carbonate were required. Possibly the most common type of waste produced worldwide is rice husk. When rice husk is heated to high

**Table 3.2** Hydroxyapatite derived from natural sources

| Sources | Extraction process | Phase | Crystallinity | Shape | References |
|---|---|---|---|---|---|
| Bighead carp (*Aristichthys nobilis*) scales | Deep eutectic solvents | HAp | 43.13% | Irregular | Liu et al. (2020) |
| *Rapana thomasiana* shells | Hydrothermal method | HAp | 79% | Irregularly shaped microcrystalline aggregates | Madalina Cursaru et al. (2022) |
| Eggshell | Hydrothermal method | HAp | Not available | Highly agglomerated with no regular shape | Oladele et al. (2019) |
| Chicken bone Black Sumatra Fighting cock | Thermal annelation (700, 900, 1100 °C) | HAp | 2.348544 0.762829 | Porous structure | Vinoth Kumar et al. (2021) |
| *Meretrix meretrix* clam shells | Calcination (900 °C) followed by a wet precipitation reaction | HAp | 75.8% | Spherical shape Average particle size 40 nm | Sindhya et al. (2022) |
| *Anadara granosa* waste | Calcination (900 °C) followed by a wet precipitation reaction | HAp | Not available | Aggregated particles with a size range of 65–48 nm have cubic form | Dhanaraj and Suresh (2018) |
| Human<br>Bovine<br>Porcine | Calcination at 600, 900, 1200 °C | HAp | 96%<br>95%<br>96% | Not available | Figueiredo et al. (2010) |
| Bovine | Treatment in aqueous NaOH and KOH solutions | HAp | Not available | Rectangular shape | Brzezińska-Miecznik et al. (2015) |
| Catfish bone<br>Tilapia bone<br>Seabass bone<br>Yellowfin tuna | Calcination at 700 °C | HAp<br>HAp<br>HAp<br>HAp | Highly crystalline | Nonuniform particle<br>Uniform particles (150 nm)<br>Fine particles (50–70 nm)<br>Uniform particles (150 nm) | Nam et al. (2019) |

(continued)

**Table 3.2** (continued)

| Sources | Extraction process | Phase | Crystallinity | Shape | References |
|---|---|---|---|---|---|
| Bovine bone | Subcritical water process<br>Alkaline hydrothermal hydrolysis<br>Thermal decomposition (850 °C) | HAp<br>HAp<br>HAp | Not available<br>Poor crystallinity<br>Highly crystalline | Nanorod shape<br>Nano flake shape<br>Nonuniform agglomerated big particle | Barakat et al. (2008) |
| Atlantic cod fish bones | Calcination (700–1200 °C) | HAp, β-TCP | Not available | Spherical-shaped (100–200 nm diameter) | Piccirillo et al. (2015) |

temperatures, it can be easily removed to create rice husk ash (RHA), which contains a significant quantity of silica (Bakar et al. 2016). Additionally, millions of tons of eggshells are produced as biowaste. When eggshells are heated to a high temperature, they produce 99% or more calcium oxide and a small amount of biologically beneficial ions like $Mg^{2+}$ and $Sr^{2+}$ (Jayasree et al. 2017). Additionally, the composition displays antibacterial properties for calcium oxide extracted from eggshells. Therefore, using biological forms of silica and calcium oxides to synthesize biomaterials for biomedical purposes decreases total manufacturing costs and offers several beneficial biological impacts (Palakurthy et al. 2020).

Employing biowastes like RHA and eggshells, Palakurthy et al. used the melt-quenching process to create $SiO_2$-CaO-$Na_2O$ bioglass (Palakurthy et al. 2020). After 7 days of SBF incubation, the $SiO_2$-CaO-$Na_2O$ bioglass displayed significant bioactivity as evidenced by the deposition of a hydroxyapatite on surface. The study effectively showed that RHA and eggshells are possible beneficial, affordable alternatives to CaO and $SiO_2$ to prepare bioglasses.

By using the sol-gel process, Nayak et al. isolated sodium silicate from RHA for use in the fabrication of BG (soda-lime $SiO_2$ based) (Nayak et al. 2010). Gel powder underwent a 2-h calcination process at 700 °C to create BG powder. Crystalline Combeite-I, distributed in an amorphous glass matrix, makes up the majority of the calcined BG powder. It was discovered that the crystalline combeite phase of glass-ceramics dissolves easily in SBF and Tris buffer solution. The glass-ceramics surface began to form carbonated hydroxyapatite after only 3 days of incubation in SBF. Using a simple, low-cost extraction method, Kaya et al. extracted novel porous biosilica-based beads from the marine sponge *Geodia macandrewii* (Kaya et al. 2021). According to bioactivity investigations, hydroxyapatite developed on the beads' surface. Additionally, employing the biosilica beads as controlled cargo release carriers for anticancer drugs is encouraged by the hollow routes leading to the center cavity (Fig. 3.2).

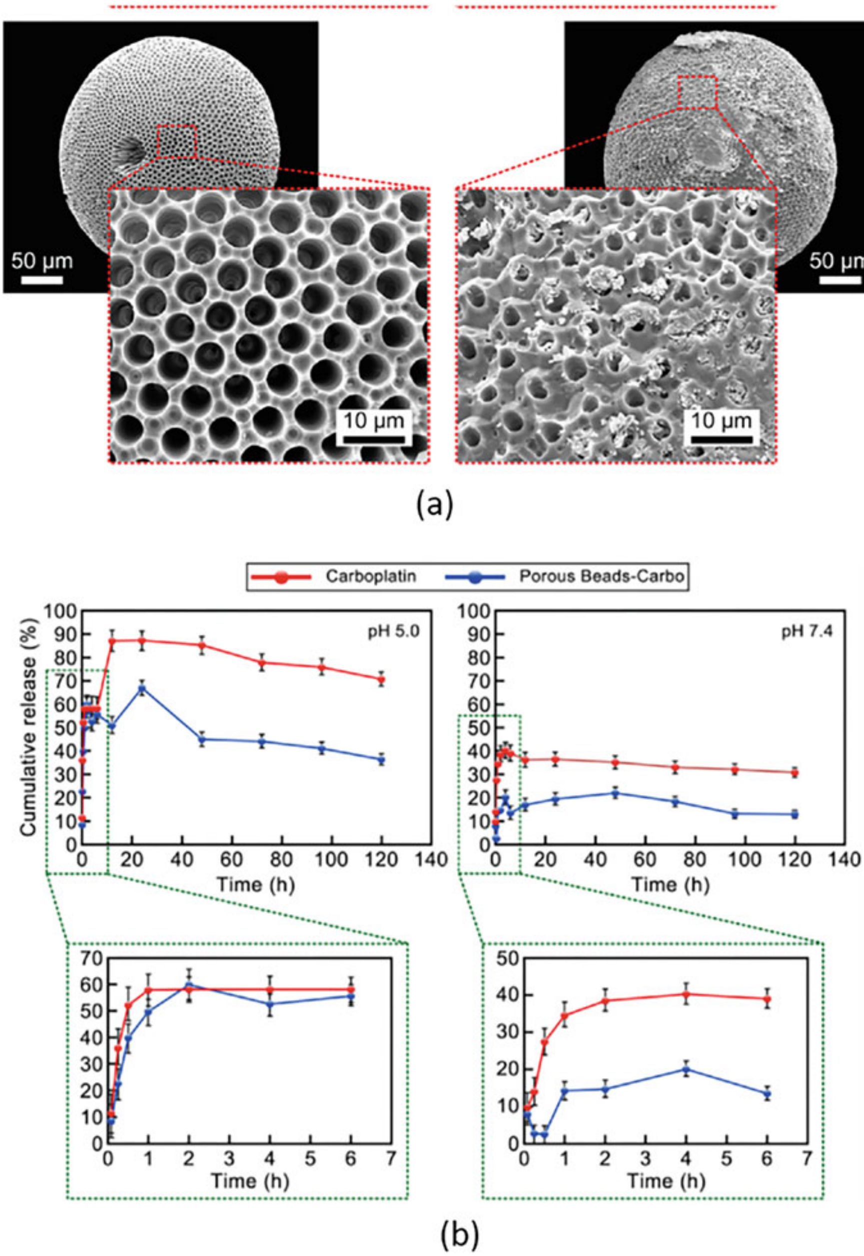


**Fig. 3.2** (**a**) SEM images of porous biosilica beads with carboplatin loaded (right) and unloaded (left). (**b**) Study on medication release between pH 5.0 and 7.4 (Kaya et al. 2021). (Reproduced from open access publication)

### 3.3.3 Collagen

The main extracellular protein in our body is collagen, which helps to protect tissues and organs mechanically. Following hydroxyapatite as the second-largest component by weight, collagen makes up 36% of bone. Type-I collagen is available in our skin, tendons, and ligament bones. Three tropocollagen molecules (Gly-X-Y-) are found in collagen, where X is proline and Y is hydroxyproline residue. The protein can create triple helical shapes because of its structure (Shoulders and Raines 2009). Collagen extraction is expanding daily due to its growing demand and use in dental and tissue engineering applications, bone graft, food and pharmaceutical industries, and more. Currently, 29 different kinds of collagen have been recognized. Type-I is found in the ligaments, bones, and tendons; type-II is found in cartilage and vitreous body; type-III is found in reticular fibers of the lungs, liver, vessel wall, and spleen; type-IV is found in basement membranes; and type-V is distributed along with type-I (cornea). Because type-I collagen has a greater capacity for cell adhesion and is less antigenic than the other forms, it has mostly been used in biomedicine for wound dressing, tissue engineering construction, and cosmetics (Ahmed et al. 2020).

Collagen is mostly found in the skin and bone of bovine species, including cows, oxen, buffalo, and cattle. Bovine collagen that has been hydrolyzed and taken from various tissues exhibits antibacterial, antioxidant, and antihypertensive properties. Bovine lung hydrolyzed collagen also exhibited anti-inflammatory and antioxidant properties. Due to the spread of numerous illnesses like infection in the mouth and foot, transmissible spongiform encephalopathy, and bovine spongiform encephalopathy, the collagen from this origin is not extensively used.

Porcine skin and bones are also employed as sources of collagen. The majority of the uses for these sources are industrial. This type of collagen is identical to human collagen; it is regarded as safe because it cannot elicit any adverse reaction. Two essential phases are involved in collagen extraction: (1) pretreatment of starting material and (2) collagen isolation. Prior to collagen isolation, the primary goal of pretreatment is to eliminate contaminants. Skin, bone, swim bladders, and scales were among the by-products that were divided into several groups prior to pretreatment of the raw materials. This facilitates quick cleanup, contamination cleanup, and size reduction. Most of the time, fats, pigments, and other proteins are present in the raw materials utilized for extraction. Other inorganic substances, including calcium, are also found in fish bones and scales. These bones and scales are typically demineralized using inorganic materials like EDTA (ethylene diamine tetra-acetic acid). Sodium chloride, sodium hydrochloride, and *n*-butanol are used to remove the non-collagenous parts and lipids. Collagens are extracted using a variety of techniques, and according to these techniques, they can be divided into four categories: acid-soluble collagen (ASC), salt-soluble collagen (SSC), ultrasound-aided collagen (UAC), and pepsin-soluble collagen (PSC). Depending on the type of extraction procedures used, different collagens have different yields and

**Table 3.3** Summary of literature about the source, extraction method, and collagen yield

| Source | Method used | Yield | References |
|---|---|---|---|
| Skin of *Centrolophus niger* (black ruff) | ASC method | 25%, 32%, 31%, 31%, and 45% ASC, respectively, were produced by acetic, citric, tartaric, formic, and lactic acid | Bhuimbar et al. (2019) |
| Tilapia skin | Acetic acid method<br>Hot water method<br>Sodium hydroxide method | 11.7%<br>10.7%<br>8.5% | Bi et al. (2019) |
| Snakehead skins | PSC extraction | 34.91–48.17% | Liu et al. (2019) |
| Sheep<br>Lamb | ASC extraction | 18.5%<br>12.5% | Vidal et al. (2020) |
| Chicken sternal cartilage | Type-II pepsin-soluble collagen subjected to ultrasonic treatment | 40% | Akram and Zhang (2020) |
| Silver carp fish skin by-product | ASC<br>PSC | 42.85%<br>58.75% | Faralizadeh et al. (2021) |
| Asian bullfrog (*Rana tigerina*) skin | ASC<br>PSC | 22.48–31.12%<br>22.59–28.30% | Indriani et al. (2022) |
| Nile tilapia skin (*Oreochromis niloticus*) | ASC | 15.3–18.3% | Menezes et al. (2020) |
| Common starfish | ASC (pretreatment steps employed ultrasound (US) and high shear mechanical homogenization (HSMH)) | 80.30 ± 0.61, (collagen extracted by classic method)<br>81.47 ± 0.45 (collagen extracted with the aid of HSMH)<br>83.18 ± 1.74% (collagen extracted with the aid of HSMH and US) | Vate et al. (2022) |

physiochemical characteristics (Table 3.3). Bhuimbar et al. (col1) extracted ASC from the skin of *Centrolophus niger* (black ruff). Figure 3.3 from their research demonstrates the impact of various acids and time on yield. The pretreated skin was given a 72-h treatment with 0.5 M lactic acid to achieve the highest yield.

Liu et al. (col 3) used snakehead skin to extract collagen and studied the effect of $H_2O_2$ pretreatment and pepsin hydrolysis strategies. They reported that both the parameters of the pepsin hydrolysis and $H_2O_2$ pretreatment processes substantially influenced the production of collagen from snakehead skin. The pH of $H_2O_2$ bleaching significantly affects the color and structure of PSC.

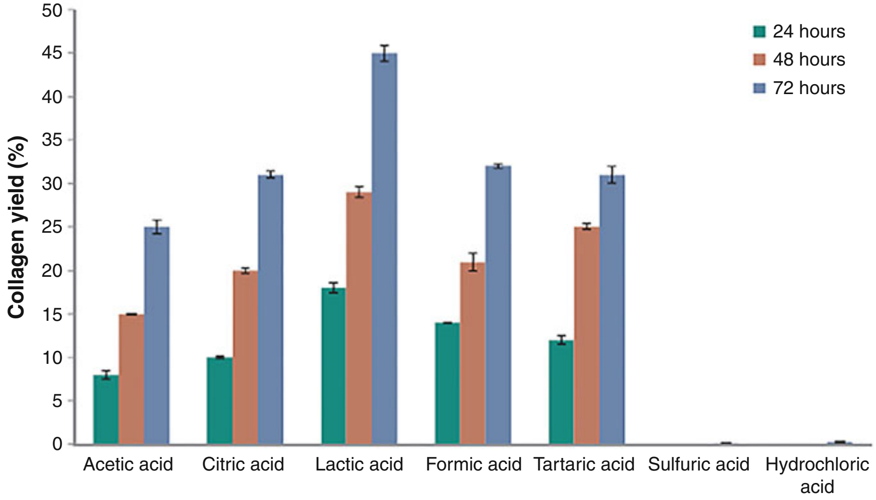


**Fig. 3.3** Effects of different acids and time on collage yield. (Reproduced with permission from Bhuimbar et al. 2019)

### 3.3.4 Chitosan

The second most prevalent natural polymer after cellulose is chitin, which is made up of a linear chain constructed of poly (1,4) *N*-acetyl-D-glucosamine (Rinaudo 2006). Chitin makes up the majority of the exoskeletons seen on marine creatures and insect shells. Biopolymers called chitin and chitosan are derived from many terrestrial and aquatic resources, such as grasshoppers, insects, fungi, shrimp, crab, conus, lobster, and squid pens (Jantzen da Silva Lucas et al. 2021; Kumar and Shahid 2020; Sayari et al. 2016). Chitin's crystal structure was dependent on the arrangement of polymer chains, mainly where the 2-*N*,*N*′-diacetylchitobiose units were located in relation to one another. Chitin has been divided into three distinct forms based on the crystalline allomorphs: α, β, and γ. The α-chitin are made of antiparallel chains, β-chitin consist of parallel chains, and γ-chitin are made of parallel and antiparallel chains (Kaya et al. 2017). The reaction conditions, chitin source, and degree of deacetylation are the factors that influence the molecular weight of chitosan. Slight variations in these parameters can have a major impact on the characteristics of chitosan, like surface properties, solubility, cellular activity, and crystallinity (El Knidri et al. 2018; Qinna et al. 2015). The surface morphology of chitosan extracted from shrimp shells was significantly affected by the concentration of acid, as reported by Hisham et al. (2021). Demineralization, deproteinization, and deacetylation are the three fundamental steps for the isolation of chitosan. Most of the crustacean shells are made of chitin, protein, minerals, etc., but they may also have carotenoid colors. In order to eliminate these carotenoid colors, another step, i.e., the decolorization process, can be added. This process makes use of several

**Table 3.4** Different natural sources for extraction of collagen

| Source | Process | Degree of deacetylation | References |
|---|---|---|---|
| Fungal source (*Ganoderma lucidum* spore powders) | Dual-frequency ultrasound irradiation | 81.1–81.3% | Zhu et al. (2019) |
| Antarctic krill (*Euphausia superba*) | Treatment in 1.7 M HCl at room temperature for 6 h, followed by 1 h of 2.5 M NaOH treatment at 75 °C | 11.28 ± 0.86 | Wang et al. (2013) |
| Fungal biomass of *Rhizopus oryzae* NRRL1526 | Microwave-assisted extraction | 94.6 ± 0.9% | Sebastian et al. (2019) |
| Shrimp shell powder | Co-fermentation involving *Acetobacter pasteurianus* and *Bacillus subtilis* | 19.6% | Zhang et al. (2021) |
| Two common spider species (*Geolycosa vultuosa* and *Hogna radiata*) | Treatment in 4 M HCl solutions and 1 M NaOH solutions | 97–99% | Kaya et al. (2014) |
| Shrimp shells | Enzymatic deproteinization | 9% | Younes et al. (2014) |
| Antarctic krill shell | Using lactic acid and dispase | 80.8% | Yu et al. (2020) |

inorganic and organic solvents like sodium hypochlorite, hydrogen peroxide, and acetone (El Knidri et al. 2018) (Table 3.4).

### 3.3.5 Hyaluronic Acid

Hyaluronic acid (HA) is the only non-sulfated glycosaminoglycan found in almost all extracellular matrix (approximately 0.5 mg/mL in human body ECM) (Becker et al. 2009) of soft connective tissues. The structural basis of HA is a linear anionic polysaccharide chain of alternative disaccharide units composed of β (1,4) glucuronic acid and *N*-acetylglucosamine (GlcNAc). It has been differentiated as higher molecular weight HA (more than 100 kDa) and low molecular weight HA (below 100 kDa); the former is denser as compared to the latter forms, and it is prevalently used in bone resorption rate modification and to form tissue scaffolds with optimized mechanical stability. This compound is also found in plants. The concentration of hyaluronic acid in animal tissues varies based on their composition and biochemical pathways. HA of higher molecular weight gives the scaffold viscoelasticity and helps to stimulate the chondrocyte for regeneration. It also increases the ECM synthesis in the tissue by preventing cell proliferation. Due to its non-immunogenicity, biocompatibility, and high water-retention capacity, it has become helpful in tissue engineering (Sionkowska et al. 2020). This also has various biomedical and pharmaceutical applications such as lubrication medium, cell-signaling factor, and other biophysical properties. The natural synthesis of hyaluronic acid in the body occurs with the help of hyaluronan synthase, degraded by hyaluronidase, and bound proteins are digestible by protease.

The synthesis of hyaluronic acid is developed by the extraction from marine sources and cattle animal tissues or fermentation process or enzymatic process and biosynthesis from different bacterial strains like *Streptococci* sp., *Corynebacterium* sp., etc. (Fig. 3.3) (Boeriu et al. 2013; Srisantisaeng et al. 2013). It was first discovered and extracted from the vitreous humor of bovine eyes in 1934 by Meyer et al. The key steps of synthesis are extraction and purification; depending on the ratio of these two steps, the property of HA is determined (Karami et al. 2021). Generally, a range of molecular weight varies from 20,000 to $13 \times 10^6$ Da, which forms the nature of its activity (Karami et al. 2021). The weight modifications by different elongation systems due to alternative purification processes mainly cause variations in rheological characteristics of HA, such as viscosity and elasticity. Usually, the method for the HA extraction includes both enzymes and organic solvents like ethanol, acetone, and propanol. After extraction, processing, and purification were carried out through physical separation methods (such as ultrafiltration, electrodeposition of protein, or adsorption) to separate HA from the broth. Biotechnological processing for commercial production of HA is drawing more attention due to the high yield and high purity over some time. Animal-derived HA is mainly used for industrial production purposes where the purity is less than that of bacterial (Fallacara et al. 2018). Sources used so far in various researches include tissues from cattle, pigs, rabbits, fish, chickens, etc. Table 3.5 describes a few natural sources used in different studies made on HA production so far. One major source used for extraction is synovial fluid and cartilage, which is an important component in a matrix composed of collagen and hyaluronan. Some other principal waste parts are chicken crest or comb, eggshells, human umbilical cord, visceral tissues, bovine and fish vitreous humor, and ovine synovial fluid (Abdallah et al. 2020; Ibragimova et al. 2022). The primary goal was to obtain the optimum concentration of hyaluronic acid possible from animal waste or by-products to reduce production costs (Rossatto et al. 2022).

The chicken comb is the most commonly available source of hyaluronic acid. Various reports have shown that this production method requires cheaper setup and chemicals. Although there are chances of immunological activity and other binding proteins, the presence of viruses (Kang et al. 2010; Murado et al. 2012), through this procedure, significant yields have been obtained. An extraction process by (Kulkarni et al. 2018) has followed the isolation process through delipidation and dehydration before obtaining protein-eliminated sodium hyaluronate powder. The confirmatory tests for protein removal and SDS-PAGE have shown that the final yield of HA was relatively pure for clinical and research applications. The viscosity measured in HA was 2.55 poise. The reason for using sodium citrate was to separate extracted HA from sodium hyaluronate. Similar strategies were taken in other reports, but the purification strategy determines the product purity. To obtain 1600–2000 kDa[1] of

[1] kDa: kilo Dalton

**Table 3.5** Various sources used for A synthesis

| Source animal tissue/microbes | | Extraction process | Purity | References |
|---|---|---|---|---|
| Poultry | Chicken comb | Organic solvent | Standard | Kang et al. (2010), Tovar et al. (2012) |
| | Eggshells | | | |
| Animal | Mice | Organic solvent | Moderate | Lambe et al. (2021) |
| | Porcine | | Moderate | Abdallah et al. (2020) |
| | Bovine synovial fluid | Chemical, enzymatic | Moderate | Abdallah et al. (2020) |
| | Bovine nasal cartilage | Organic solvent | | Boeriu et al. (2013), Lambe et al. (2021) |
| | Sheep synovial fluid | Enzymatic, solvent extraction | Standard | Abdallah et al. (2020), Ibragimova et al. (2022), Lambe et al. (2021) |
| | Sheep lung | | | |
| | Rabbit liver, cortex, vitreous, lung | | | |
| Human | Umbilical cord, placenta, visceral fluid, serum, epidermis | Enzymatic, solvent extraction | Moderate | Fallacara et al. (2018), Abdallah et al. (2020), Ibragimova et al. (2022), Lambe et al. (2021) |
| Marine sources | Fish eyes | Chemical, organic solvent, enzymatic separation | High | Abdallah et al. (2020), Murado et al. (2012), Lambe et al. (2021) |
| | Fish scales | | | |
| | Fish extracts | | | |
| Microbial sources | *Streptococcus* sp., *Bacillus*, *E. coli*, *Corynebacterium*, *L. acidophilus*, and yeasts (saccharomyces, *Pichia pastoris*) | Enzymatic, batch culture, genetic manipulation | High | Karami et al. (2021), Fallacara et al. (2018), Rossatto et al. (2022), Rodriguez-Marquez et al. (2022) |

HA, Murado et al. used marine fish eyeballs (vitreous humor) with consecutive separation and purification through enzymatic digestion and chemical processes (Murado et al. 2012).

Enzymes like papain, trypsin, pepsin, and proteases are essential for the degradation of tissue. It helps to break the glycosidic bonds of the total protein to isolate the undamaged HA polymer from raw materials. Among these, papain is the most used enzyme for HA isolation. In a study, enzymatic hydrolysis is where enzymes such as papain, pepsin, and trypsin were applied on eggshell membranes to find the ideal physicochemical conditions like temperature and pH for extracting high molecular weight HA (Ibragimova et al. 2022). Microbial fermentation: The most promising source for bacterial hyaluronic acid production is the *Streptococcaceae* family, and among them, strains of *Streptococcus zooepidemicus* have given the highest yield (6 g/L) of HA as reported in some research (Ibragimova et al. 2022; Rodriguez-Marquez et al. 2022). As there is a difference in the extraction process for high

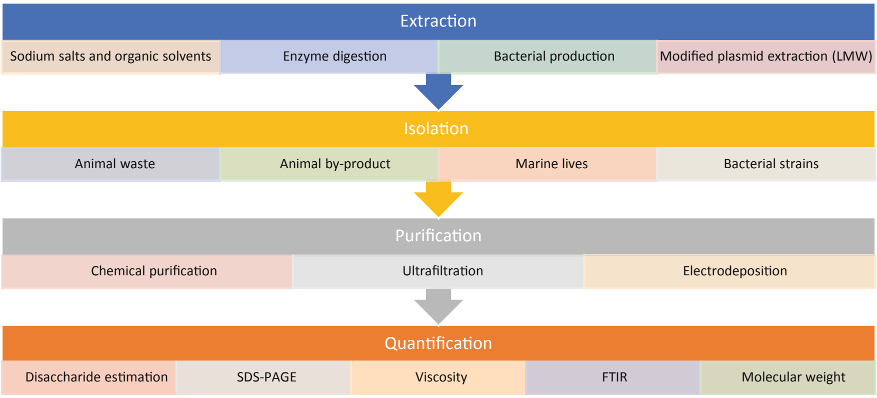


**Fig. 3.4** Various methods for HA synthesis

molecular weight HA and low molecular weight HA, microbial fermentation can also be modified to obtain the desired HA. Using *Lactococcus acidophilus* HA synthesis was safe as compared to *Streptococcus zooepidemicus* due to the genetic manipulation (Rossatto et al. 2022), but it does not cut down the cost of production either. Nowadays genetically modified strains of microbes are used such as *Streptococcus thermophilus* YIT 2084, *Streptococcus zooepidemicus* ATCC 35426, ATCC 35246, *Corynebacterium glutamicum*, recombinant *Bacillus*, etc. The rate of production can be increased by using these genetically modified bacteria, but the use of enzyme inhibitors bypassing the genetic change also helps to increase the rate as stated by Samadi et al. (2022). Another study shows that *Streptococcus zooepidemicus* alters the production rate when 0.15 g/L of hyaluronidase is added in bacterial culture and thus increased dissolved oxygen concentration to enhance HA yield (6 g/L) more compared to yield without enzyme addition (5 g/L). Albeit the increase in yield, it has decreased the molecular weight from 1300 kDa to 45 kDa (Karami et al. 2021; Samadi et al. 2022) (Fig. 3.4).

### 3.3.6 Cellulose

One of the naturally derived fibers is cellulose. Generally, it is found as a fiber composite with hemicellulose, lignin, and other protein molecules. This most abundant biopolymer of nature is increasingly being used in tissue engineering, as a scaffold component, in substrate preparation, and for producing other composite nano-polymers. These scaffolds can be suitable material for 3D tissue formation (nerve inside the bone tissue) and cell growth for having surface modification properties and mechanical stability (Hickey and Pelling 2019). Cellulose is abundant in many forms. Most of it comes from wood and plant sources like plant fibers such as jute, hemp, kenaf, and cotton (Fig. 3.5). Other than the direct plant sources, agricultural wastes, composite biomass, and processing residues from industries also

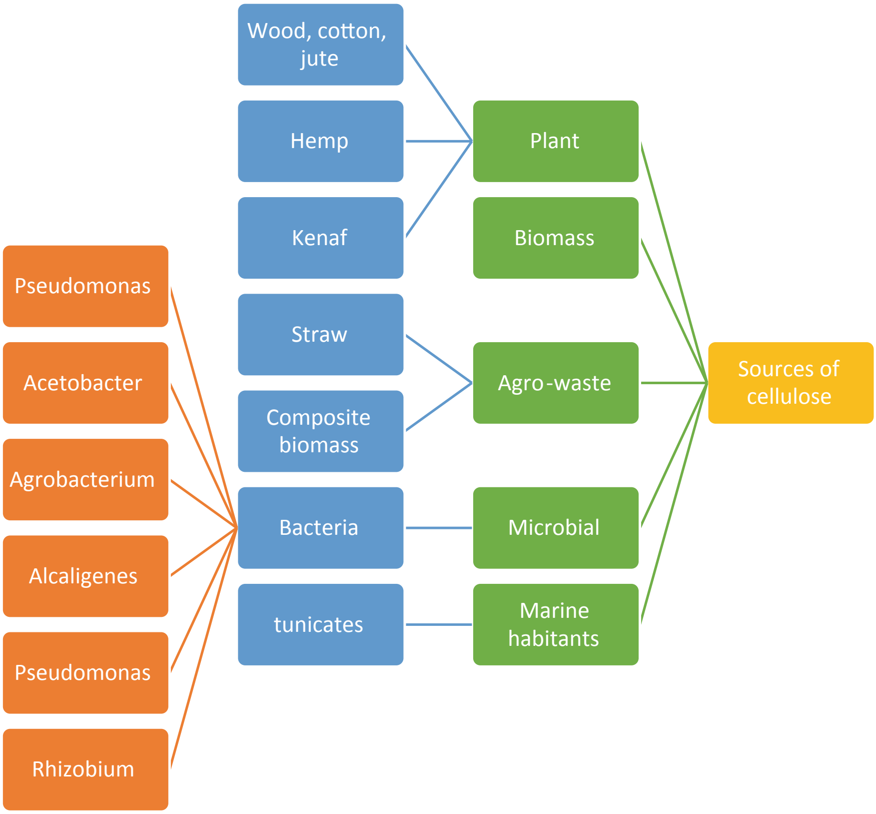


**Fig. 3.5** Different common sources of cellulose

contain a large amount of cellulose. The microbial sources of cellulose are mostly bacteria. Beside this, marine biomass also has plenty of cellulose sources. The functional characteristics of cellulose depend on its chain length, polymerized units, and presence of glucose that forms molecular units of cellulose. Different forms of cellulose are shown in Fig. 3.5. The structural basis is cellulose fibril, which resulted from biosynthesis. The outline of the cellulose extraction from sources other than bacteria is described in Fig. 3.6.

The application of cellulose and its derivatives is widespread, and currently, researchers are focusing on the production and dissociation of cellulose nanoparticles (CNP) (Jonoobi et al. 2015). These are highly applicable to drug delivery systems as bacterial cellulose offers localized delivery systems in cells by means of increasing cytokine concentration. There is a similarity between cellulose fibers and collagen fibers of bone tissue; this can be utilized in bone tissue engineering. It is also found in many studies that the biocompatible scaffolds composed of cellulosic composites enhance bone regeneration (Hickey and Pelling 2019; Seddiqi

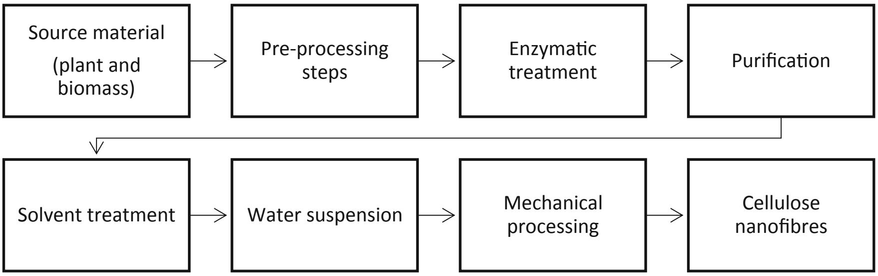


**Fig. 3.6** Stepwise process of nanoscale cellulose fiber production (Jonoobi et al. 2015)

et al. 2021; Teeri et al. 2007). It also stimulates calcium deposition, and hydroxyapatite composites with cellulose nanocrystals allow rapid bone integration.

### 3.3.7 Starch

Starch is a biodegradable polymer primarily available in the cell walls of plants. This polysaccharide is composed mostly of amyloses, a linear polymer of D-glucose units attached through α-1,4 glycosidic linkage, and amylopectin, a branched polymer of D-glucose units joined by α-1,4 bonds and cross-links at each 25–30 glucose units joined by α-1,6 bonds. The use of starch is determined by the branching patterns of its units, and the physicochemical properties like higher water affinity and low tensile strength restrict its uses. Starch is a good candidate among biopolymers to produce biopolymer products for short-term stability like molded parts and bone scaffolds that need to be biologically active and degradable. For preparing biocomposites, starch can be mixed with other polymers as well as raw materials (such as cassava tree bark and gelatinized starch) to enhance the physicochemical properties of the desired polymer or scaffold (Engel et al. 2021). Also, it has high requirements in the food and food processing industries, where amylose and amylopectin are extracted as an additive in food processing.

Different plant sources are widely used for starch production, among which corn starch is mostly known and produced (about 90–95% of total starch production) (Shevkani et al. 2016). Though fruits and food grains (except corn) are less common in the production route for their food value, rather a novel approach using rotten and waste fruits has been recently tested where they have utilized a variety of wasted fruits like kiwifruit, avocado, cassava, mango, litchi, tamarind, annatto, jackfruit, apple, pineapple, banana, etc. (Kringel et al. 2020); a common production method is shown in Fig. 3.7. Currently, alternative extraction methods are developed to replace conventional maceration, as the new approach has better acceptance in terms of eco-friendly procedure and faster production using the ultrasound-assisted extraction system (Setyaningsih et al. 2021). The UAE[2] technique uses ultrasonic waves to

[2]UAE: ultrasonic assisted extraction

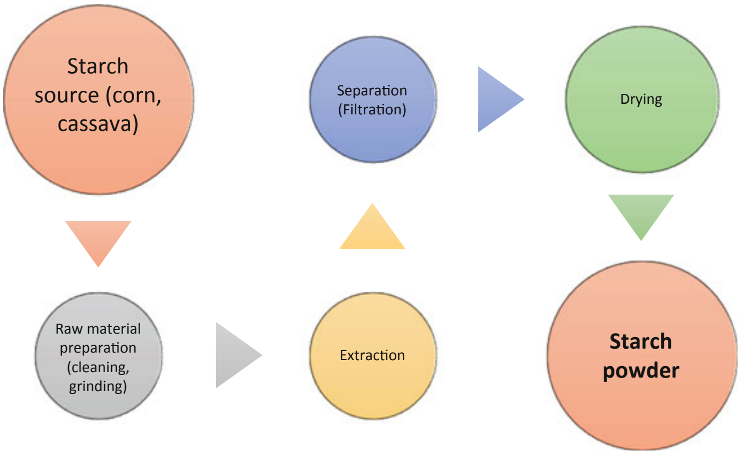


**Fig. 3.7** Starch production method. (Adapted from Akram and Ahmed 2014; Madalina Cursaru et al. 2022)

produce a cavitation effect through a medium. As a result, the cell walls are broken down and allowed to release in the solvent medium. Because the starch granules in cassava are trapped by cellulose fibers compacted with pectin inside the cell wall, another study has shown that sago (*Metroxylon sagu*) and taro (*Colocasia esculenta*) are very promising alternatives for starch production (Ishak et al. 2020).

### 3.3.8 Alginate

Alginate polymer is abundant in nature, a polysaccharide of two isomer residues, β-D-mannuronic acid (M) and α-L-guluronic acid (G) with 1,4 glycosidic bonds in three different conformations (homogeneously or heterogeneously) of poly mannuronic (M) blocks, poly guluronic (G) blocks, and blocks of MG isomers. It is predominantly present in various algae (mostly brown and red algae), bacteria (*Azotobacter*, *Pseudomonas*, etc.), and plants. Depending on the sources and the growth conditions, the structural and functional properties of alginate vary in different residual parts.

Numerous industries have made substantial use of alginate-like food and bioprocess industries, as well as in biomaterial applications, due to their high stability and biocompatibility. The production of alginate also has a commercial background due to its popular application. Besides its physicochemical properties, it has potentially been used in pharmaceutical areas. Currently, more acceptable derivatives from alginate are gaining attention due to the environmental acceptance and easier extraction methods. The degradation requires enzymes to perform rapid production of alginate oligosaccharide and desired degree of polymerization for desired mechanical and chemical properties (Cheng et al. 2020; Kivilcimdan Moral and Yildiz 2016). The effect of sources used for production through bacterial synthesis determines the purity, cost, and yield of the final product. The culture medium has also influenced the rate of output per batch. To eliminate the wastage of pure materials during processing, cheaper carbo sources can be utilized as a nutrient. One report has shown that molasses, a by-product of the sugar manufacturing process, can be an alternative as a carbon source and also helps to enhance the rate, but it is not used in commercial fields. Starch is an effluent material in other biopolymer production processes, and it can be utilized to reduce cost also (Kivilcimdan Moral and Yildiz 2016).

## 3.4 Challenges to the Use of Materials from Renewable Resources

The fundamental challenge in biologically derived compounds compared to synthetically or chemically produced materials is the impurity in composition and concentration. The downstream processing of materials causes high prices for lower yields of products. To eliminate this problem, comparatively new synthesis methods are being experimented with where the post-extraction procedure should be less (Rossatto et al. 2022). Also, there is significantly less amount of information about the compositions and advantages of using animal and agricultural wastes and by-products. As a result, a huge percentage of raw materials are almost out of the processing circle. Proper channeling of these materials can save resources and produce more such polymers in an eco-friendly way (Motaung and Linganiso 2018). The aspects of production challenges are shown in Fig. 3.8. For their optimal use, it is imperative to explore these abundant marine biomaterials in the study. Researchers have recently given a great deal of attention to its widespread use in

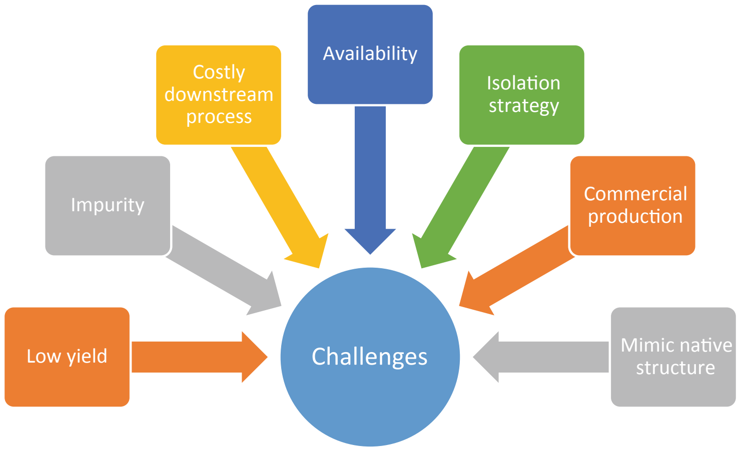


**Fig. 3.8** Challenges in material production

numerous applications. These biomaterials are essential for bone tissue engineering because they are affordable, secure, and biocompatible (Lalzawmliana et al. 2019).

In the case of hydroxyapatite, excellent performance has been demonstrated despite the limited number of comparison studies that are currently available. The optimal tissue engineering scaffolds using hydroxyapatite as the nano-building block still present considerable challenges, despite substantial advancements in the development of materials with sufficient mechanical strength. The development of various hydroxyapatite-based scaffolds will also be suggested using fresh biomimetic or bioinspired methods. In comparison with marine sources, extracting chitin from insect biomass is clearly more difficult. Green technologies or process optimization may result in items that are produced in large quantities, but this may only be done after substantial research (Mohan et al. 2020). In the case of hyaluronic acid, its production faces multifaceted issues like a low amount of final product from bulk animal waste as a report shows, though the purity was 99.4% (Murado et al. 2012). This indicates that the downstream processing cost cannot be avoided for bulk raw materials. Another challenge is the isolation and enzyme digestion. For microbial production procedures, contamination is a primary issue, and maintaining culture for a long time also has drawbacks, such as unexpected enzymatic degradation or molecular weight degradation (Ibragimova et al. 2022; Samadi et al. 2022). In the case of cellulose, the commercial production from these plant residues has not been encouraged much as there is no established detailed isolation and processing method in the current research scenario, and it is less known to a large part of consumers. Agricultural wastage management is not adequately formulated for cellulose production, as found in a few papers (Motaung and Linganiso 2018).

Starch production from conventional sources creates environmental problems due to the formation of methane, and low yield from cassava is also a problem. However, it is still a commercially used process (Ishak et al. 2020). The raw plant sources, without proper treatment, may give side products like methane and other starch composites, which are not desirable for production (Kivilcimdan Moral and Yildiz 2016; Rojas-bringas et al. 2021). For alginate production, by-products of other biopolymers like starch wastes are also used, where impurities are more significant. In some methods, direct plant or animal sources have been used where the chance of side products increases due to minimized downstream processing (Kringel et al. 2020; Kivilcimdan Moral and Yildiz 2016). Preclinical tests and extensive studies on bone tissue engineering have been conducted and are still being done. The goal of all of these studies is to create the perfect bone tissue engineering scaffold that will meet the basic needs of a bone substitute, including high mechanical strength with adequate elasticity, perfect biocompatibility to support cell adhesion, migration and proliferation, sufficient porosity for facilitate nutrient and oxygen transfer, the ideal degradation rate to support the formation of new bone and cartilage, and controlled release of growth factors.

## 3.5 Conclusion

Bone tissue engineering is an appropriate substitute for autograft and allograft to treat bone defects. The design of scaffolds using biomaterials and their synthesis are an important part of bone tissue engineering. The characteristics of the biomaterials utilized determine whether an implant will be successful. Naturally, origin biomaterials are more advantageous compared to synthetic materials because of biocompatibility, biodegradability, and nontoxicity. Natural biomaterials are still being explored extensively for their possible applications, despite the fact that the existing results do not entirely meet clinical needs. As a result, research in this area is currently being conducted all over the world.

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