1.1 The genus *Aureobasidium*

The yeast-like fungi *Aureobasidum* spp. are classified into the phylum Ascomycota, the order Dothideales, and the family Aureobasidiaceae [1, 2]. Generally, the colonies of *Aureobasidium* appear creamy, yellow, or light pink at the beginning of the growth [3] and then they become blackish [4]. For this reason, *Aureobasidium* is popularly known as black yeast [5-7]. Moreover, some strains of *Aureobasidium* isolated in tropical and subtropical areas can synthesize orange, purple, yellow, and red pigments [8, 9].

Aureobasidum species exhibit diverse lifestyles as saprophytes and plant-associated endophytes [10-12]. They are widely distributed in various ecological environments such as indoor habitats [13, 14], the phyllosphere [15, 16], hypersaline waters [17-19], glacial ice [20, 21], synthetic polymer surfaces [22], aviation fuel tanks [23], deserts [24, 25], natural honey [26], and even radioactive sites [27]. The ubiquitous occurrence of *Aureobasidium* is attributed to their excellent adaptability, polyextremotolerance, and nutritional versatility [28, 29]. The genus *Aureobasidium* can display several morphological forms, mainly consisting of blastoconidia (yeast-like cells), swollen cells, chlamydospores, and hyphae (Fig. 1-1) [2, 30], which are determined by many factors such as cultivation conditions [31], species [4], nitrogen sources [32], and so on.

Initially *Aureobasidium* was classified into *A. pullulans* var. *pullulans* [33], *A. pullulans* var. *melanogenum* [34], *A. pullulans* var. *subglaciale* [30], and *A. pullulans* var. *namibiae* [30], which are based on the features of morphology, nutritional physiology, and metabolic spectrum. In 2014, the housekeeping genes in different *loci* within the whole genome sequence were utilized to reclassify these four proposed varieties as separate species: *A. pullulans*, *A. melanogenum*, *A. subglaciale*, and *A. namibiae* [35]. Additional species of *Aureobasidium* have been identified through the multilocus sequence analysis, which are *A. thailandense* [36], *A. proteae* [37], *A. aerium* [38], *A. leucospermi* [37], among others. It has been known *A. pullulans* does not reproduce sexually [2, 39]. Indeed, genome sequencing indicates that *A. melanogenum* strains are strictly clonal. In addition, the frequent diploidy in *A. melanogenum* is caused by intraspecific hybridization between haploids and is not followed by meiosis or haploidization [40].

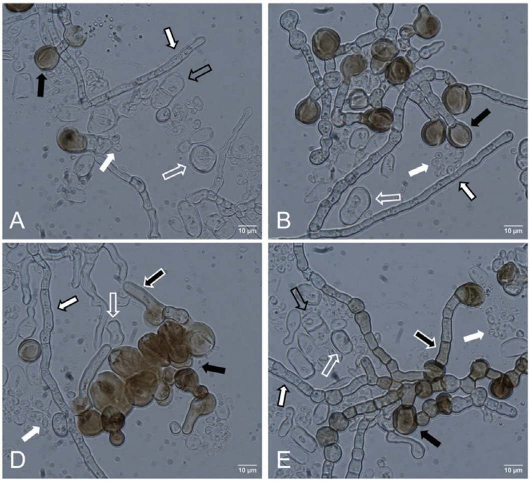

Figure 1-1. The polymorphologies of *A. pullulans* under a light microscope [41]. They include yeast-like cells or blastoconidia (white arrow), septated swollen cells (black open arrow), non-septated swollen cells (white open arrow), chlamydospores (back arrow), hyaline hyphae (black lined, white filled arrow), and dark hyphae (white lined, black filled arrow). Figure was taken with permission from [41].

1.2 The hydrolytic power of *Aureobasidium*

It has been reported that the genus *Aureobasidium* can secrete a powerful arsenal of extracellular enzymes [42, 43], including cellulases [44, 45], xylanases [8, 46-49], amylases [50, 51], lipases [52-55], and mannanases [56, 57]. This remarkable trait allows for an efficient degradation of lignocellulosic biomass and other biopolymers. For instance, *A. pullulans* LB83 displays a maximum cellulase activity of above 7 U/mL CMCase (carboxymethyl cellulase) and of above 2 U/mL FPase (filter paper cellulase) on sugarcane bagasse [45]. *A. pullulans* 98 can produce CMCase at 4.51 U (mg protein)$^{-1}$ [44]. *A. melanogenum* PBUAP46 can secrete 5.19 U/mL xylanase in an optimized production medium consisting of 3.89% (w/v) rice straw and 0.75% (w/v) $NaNO_3$ as carbon source and nitrogen source, respectively [48]. The maximum production with 82.2 U/mL xylanase can be achieved using *A. pullulans* CCT 1262 under optimized cultivation conditions [49]. The deep sea sediment isolate *A. pullulans* N13d can secrete 58.5 U/mg amylase under the condition of 1.0% peptone and 1.0% soluble starch at pH 4.0 [50]. The amylase from *A. pullulans* Cau19 through the purification of ammonium sulfate precipitation and chromatography displayed a K_m of 6.25 mg/mL and a k_{cat} of 5.0×10^2/min [51]. It was found that *A. pullulans* HN2.3, isolated from sea saltern can produce a lipase with an activity of 8 U/mL by performing a time-dependent induction strategy [54]. The lipase gene from *A. melanogenum* SRY14-3 was heterologously expressed in *Pichia pastoris* and displayed a lipase activity of 3.8 U/mL after the induction of methanol [55]. *A. pullulans* NRRL 58524 can yield a thermostable crude mannanase with 8.42 U/mL activity when culturing in 3% defatted spent coffee grounds and 0.67% ammonium sulfate [56].

A remarkably wide secretome from the genus *Aureobasidium* supports degradation or valorization of a broad spectrum of substrates such as xylose, fructose, sucrose, starch, pectin, molasses, cellulose, xylan, inulin, and other agro-based sugars and sugar polymers originating potentially from side streams [42, 58]. For example, the two strains *A. melanogenum* AS37 and SK25 can utilize xylose as sole carbon source to yield polyol lipids with different structures [59]. Fructose was used as the optimal carbon source for pullulan production by *A. pullulans* NCPS2016 [60]. Moreover, sorbitol can be converted from fructose using *A. pullulans* LP23 [61]. It was documented that sucrose can be efficiently transformed into pullulan and fructo-oligosaccharides (FOSs) using different *Aureobasidium* strains [62-64]. A melanin-deficient strain *A. pullulans* GXL-1 can yield the low-molecular-weight polymalate from liquefied corn starch *via* simultaneous saccharification and fermentation [65]. There are no reports on the valorization of pectin, but polygalacturonase, the most extensively studied pectinolytic enzyme, was detected with an activity of 8.6 U/mL in the presence of citrus pectin using *A. pullulans* P56 [66]. The mutant strain in which the glucose repressor-encoding *CREA* gene was disrupted can convert cane molasses into 0.58 g FOSs/g of molasses sugar [67]. Although cellulose as the most abundant organic material in the world has not been reported to yield commercially important metabolites using the genus *Aureobasidium*, the activities of endoglucanase and β-glucosidase were detected [44, 68]. Tropical weed xylans can be hydrolyzed by xylanases from *A. melanogenum* PBUAP46 for xylooligosaccharide production [48]. The native strain *A. melanogenum* 9-1 isolated from a mangrove environment can produce 17.4 U/mL of inulinase activity and 30 g/L crude polyol lipids in a medium containing 160 g/L inulin [69]. Agro-industrial wastes are attractive sources for the synthesis of value-added products through microbial fermentation, which may become economically viable and reduce environmental issues [70, 71]. Recently, Asian palm kernel has been reported as a more preferred substrate for producing pullulan (16 g/L) than wheat bran, rice bran, and coconut kernel [72]. *A. pullulans* URM 7059 can convert residual biodiesel glycerol into esterase cultivated in submerged fermentation [73]. Carrot peel extracts were shown as a suitable carbon source to yield 0.19 g/L intracellular and 3.52 g/L extracellular melanin by *A. pullulans* NBRC 100716 [74]. Furthermore, it has been found that *Aureobasidium* spp. show a high potential to degrade environmentally hazardous waste. For instance, it was early reported in 1995 that *A. pullulans* can grow on simple aromatic compounds including ferulic acid, catechol, protocatechuic acid, and vanillic acid [75]. *Aureobasidium* sp. derived from deep-sea oil reserves can utilize hexadecane and 1-hexadecene as the sole carbon sources [76]. *A. melanogenum* 11-1 can display a high laccase activity, which can be applied in the decolorization of synthetic dyes [77].

1.3 Natural products from *Aureobasidium*

It has been reported that the genus *Aureobasidium* is an excellent host for producing a variety of commercially attractive bioproducts such as pullulan, polymalate, polyol lipids, melanin, siderophores, fructooligosaccharides, single-cell oils, gluconic acid, fumaric acid, β-glucan, Aureobasidin A, and more [42, 78]. This introduction is mainly focused on the description of the biosynthesis of pullulan, polymalate, polyol lipids, and melanin. The biosynthetic pathways and key enzymes are schematically shown in Figure 1-2.

1.3.1 Polymalate synthesis in *Aureobasidium*

Polymalate (PMA) is a polymeric material composed of repetitive L-malic acid residues, which are connected *via* ester bonds between their α-hydroxyl and β-carboxyl groups [79-82]. PMA has some remarkable properties due to its biodegradability, water solubility, biocompatibility, non-immunogenicity, non-toxicity, absorbability, and amenable chemical modification [81-84]. Thus, PMA and its derivatives can be mainly applied in the biomedical industry as a drug delivery system and they can be processed as biodegradable plastics, detergents, malate, coating films, and other advanced materials [81, 85-88]. Notably, while for years discussed, no such commercialization happened to date.

It has been evidenced that the PMA synthetase in *A. melanogenum* is responsible for the condensation of malate into PMA [86, 89]. Furthermore, it has been proposed that malate comes from three pathways including the oxidative TCA (tricarboxylic acid) cycle, cytosolic reductive TCA pathway, and glyoxylate shunt. In the TCA cycle, oxaloacetate and acetyl-CoA are first condensed to form citric acid. After a series of oxidative reactions, fumarate is transformed into malate under the catalysis of fumarase. In the reductive TCA pathway, pyruvate is carboxylated by fixing carbon dioxide to produce oxaloacetate mediated by pyruvate carboxylase. Then, the formed oxaloacetate is reduced to malate under the catalysis of the cytosolic malate dehydrogenase. In the glyoxylate cycle, acetyl-CoA and glyoxylate are condensed to produce malate through the action of the malate synthase [90-92]. Malate is the sole precursor for PMA synthesis in *Aureobasidium* spp.

1.3.2 Pullulan – an industrial polysaccharide

Pullulan, a linear water-soluble polysaccharide, is extensively produced by various strains of *Aureobasidium*. It is mainly composed of maltotriose repeating units interconnected by α-1,6 glucosidic linkages and the internal glucose units within maltotriose are linked by α-1,4 glucosidic bond [93-96]. This regular alternating arrangement of α-1,4 and α-1,6 linkages contributes to improved solubility and structural flexibility of pullulan [97]. In addition, pullulan also exhibits highly biodegradable, biocompatible, non-toxic, non-carcinogenic, non-immunogenic, and non-mutagenic properties [98, 99]. It has wide applications in pharmaceuticals, biomedical, food, and cosmetics [100, 101].

It has been known that UDP-glucose produced from glucose-1-phosphate is a precursor for pullulan synthesis [102, 103]. The UDP-glucose is polymerized to the short chain α-1,4 glucans under the catalysis of glycogenins (Glg1 and Glg2), ceramide β-glucosyltransferase (Gcs1), and sterol glucosyltransferase (Sgt1) [104]. The intracellular glycogen synthase domain, the exopolysaccharide transport domain, and the extracellular α-amylase catalytic domain of the enzyme complex AmAgs2 are responsible for the synthesis and transmembrane transportation of long chain α-1,4 glucans, and maltotriose release, respectively. Then, the maltotriose is anchored to Lph-glucose and α-1,6 glucosidic linkages are formed between maltotrioses in the pullulan [104, 105]. It has been elucidated that glucose derepression and hyperosmolarity resistance are two critical factors for the high production of pullulan [26, 106]. Furthermore, the activities of α-amylase, glucoamylase, and isopullulanase in *A. melanogenum* can affect the molecular weight of pullulan [107, 108].

1.3.3 Polyol lipids – biosurfactants forming a heavy oil

Polyol lipids form extracellular heavy oils, which consist of a single polyol headgroup (mannitol, arabitol, xylitol, glycerol, sorbitol, galactitol, or threitol) partially *O*-acylated with three or four 3,5-dihydroxydecanoic ester groups [109-112]. Polyol lipids may have potential applications as biosurfactants; they displayed antibacterial and anticancer activity [112, 113]. It has been found that 3,5-dihydroxydecanoic acids, the tail groups of polyol lipids, are formed by acetyl-CoA or malonyl-CoA under the catalysis of a highly reduced polyketide synthase encoded by the $PKSI_{PL}$ gene. The headgroup, for instance, arabitol is contributed by arabitol dehydrogenase (ArDH) and mannitol is formed by mannitol 1-phosphate 5-dehydrogenase (MPDH) and mannitol dehydrogenase (MDH). Likely, an esterase (Est1) participates in the linkage of a single headgroup and 3,5-dihydroxydecanoyl ACP [114]. In addition, a glycolipid transfer protein encoded by the *GLTP* gene and an ABC (ATP-binding cassette) transporter encoded by the *MDR1* gene are involved in the transport and secretion of produced polyol lipids into the extracellular space [114].

1.3.4 Melanin – the molecule making the yeast black

Melanin is a hydrophobic heterogeneous and black-pigmented biopolymer but with a structure diverse and undetermined [115-117]. Melanin is involved in increasing the survival of harsh environmental conditions and pathogenicity in various fungi [24, 118]. It has been reported that melanin displays many desirable biological and physicochemical functions such as anti-oxidation, photoprotection, anti-radiation, absorption, and metal ion chelation [119, 120]. DHN-melanin (1,8-dihydroxynaphthalene) and DOPA-melanin (L-3,4-dihydroxyphenylalanine) are the two most important categories for fungal melanin [121]. The melanin produced from *A. pullulans* is mainly found as DHN-melanin [122, 123]. It has been proposed that acetyl-CoA is converted to 1,3,6,8-tetrahydroxynaphthalene (T4HN) under the catalysis of polyketide synthase encoded by the $PKSI_{M}$ gene. Then, T4HN is catalyzed to scytalone, 1,3,8-trihydroxynaphtalene (T3HN), and vermelone by T4HN reductase, scytalone dehydratase, and T3HN reductase, respectively. Eventually, vermelone is polymerized into DHN-melanin [124]. The specific transcription factor Cmr1 within the $PKSI_{M}$ biosynthetic gene cluster in the desert-derived *A. melanogenum* XJ5-1 can upregulate the expression of the $PKSI_{M}$ gene and other genes related to melanin production.

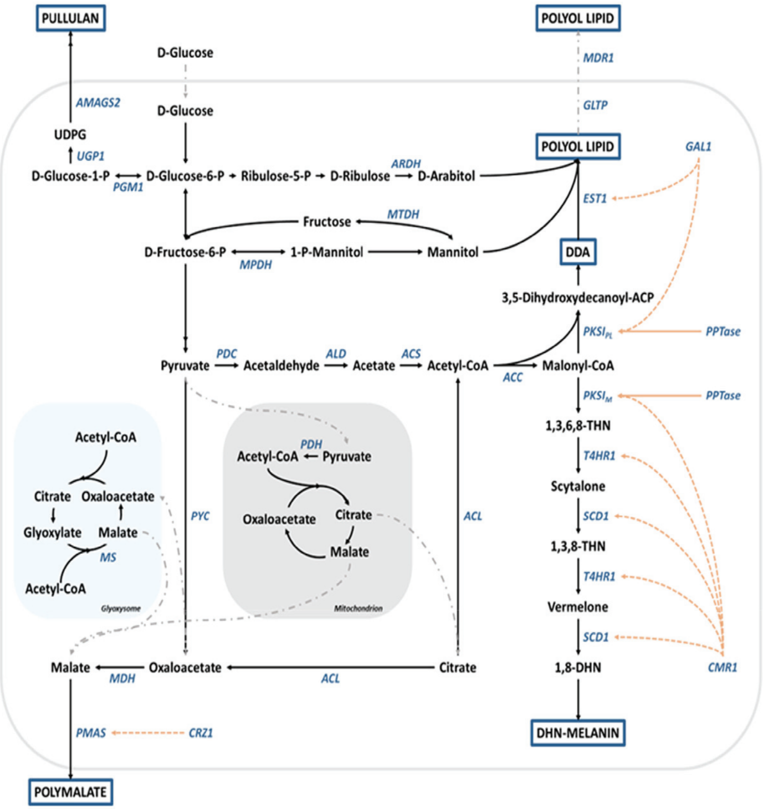

Figure 1-2. The biosynthetic pathways for pullulan, polyol lipids, polymalate, and DHN-melanin in the genus *Aureobasidium*. Genes coding for the respective enzymes are shown in blue. 1,3,6,8-THN, 1,3,6,8-tetrahydroxynaphtalene; 1,3,8-THN, 1,3,8-trihydroxynaphtalene; 1,8-DHN, 1,8-dihydroxynaphtalene; ACC, acetyl-CoA carboxylase; ACL, ATP-citrate lyase; ACP, acyl carrier protein; ACS, acetyl-CoA synthetase; ALD, acetaldehyde dehydrogenase; AMAGS2, a multidomain α-glucan synthetase; ARDH, arabitol dehydrogenase; ATP, adenosine triphosphate; CMR1, transcription factor; CoA, coenzyme A; CRZ1, transcription factor; DDA, oligo-dihydroxydecanoic acid; EST1, esterase 1; GAL1, transcription factor; GLTP, glycolipid transfer protein; MDH, malate dehydrogenase; MDR1, ATP-binding cassette transporter; MPDH, mannitol-1-phosphate dehydrogenase; MS, malate synthase; MTDH, mannitol dehydrogenase; P, phosphate; PDC, pyruvate decarboxylase; PDH, pyruvate dehydrogenase; PGM1, phosphoglucomutase 1; PKSI$_{M}$, polyketide synthase type I involved in melanin biosynthesis; PKSI$_{PL}$, polyketide synthase type I involved in polyol lipid biosynthesis; PMAS, polymalate synthetase; PPTase, phosphopantetheinyl transferase; PYC, pyruvate carboxylase; SCD1, scytalone

dehydratase 1; T4HR1, 1,3,6,8-THN/1,3,8-THN reductase; UDPG, uridine diphosphate glucose; UGP1, UDP-glucose pyrophospholase. Figure was taken with permission from [78].

1.4 Chemical and physical characteristics of PMA

PMA can be divided into three types of configurations including α-type, β-type, and α, β-type depending on the connection of the malate units (Fig. 1-3). Only β-type is the main type of PMA produced by the genus *Aureobasidium* and other microorganisms, while the α-type and α, β-type are non-natural [81, 82]. The β-type PMA has many pendant α-carboxylic groups, which can be covalently bound to various functional groups [81, 125]. Currently, PMA can be chemically synthesized by polycondensation and ring-opening polymerization [126-128]. Nevertheless, these traditional chemical methods require many reaction and purification steps, and it is neither environmentally friendly nor cost-effective [127]. PMA and its derivatives are safe macromolecules that can be used in human bodies and they display low immunogenicity and non-toxicity on normal and cancer cells [129, 130]. The aquatic solution of PMA, a strong acid, can be depolymerized into malate by heating or saponification at room temperature. The aquatic solution of 2% PMA is titrated as a polybasic acid with 3.6 of pKa and its pH value is determined as 2.0. Moreover, the ester linkages of PMA also can be transmethylated into dimethyl malate using HCl-MeOH. Free PMA is a hygroscopic glassy mass and it starts to soften at 95 to 100 °C. PMA cannot be dissolved in acetone, methanol, and other organic solvents, while it is soluble in dimethyl sulfoxide [131].

The ultraviolet spectra of PMA shows a shoulder at 205 nm, which is similar to that of malate [131]. The ^{1}H-NMR spectra of PMA has one triplet splitting (5.4 ppm, ^{1}H, CH, J = 5.96 Hz) and one doublet splitting (3.1 ppm, 2H, CH_2, J = 6.2 Hz) [88]. In addition, the IR spectrum of the methylated PMA displays absorptions at 1744 cm^{-1} (S, —C=O), 2970 cm^{-1} (S, —CH_2), and 3456 cm^{-1} (Br, —OH) [88]. These spectra data suggest that the primary ester-bond on the PMA chains produced by the genus *Aureobasidium* is β-type.

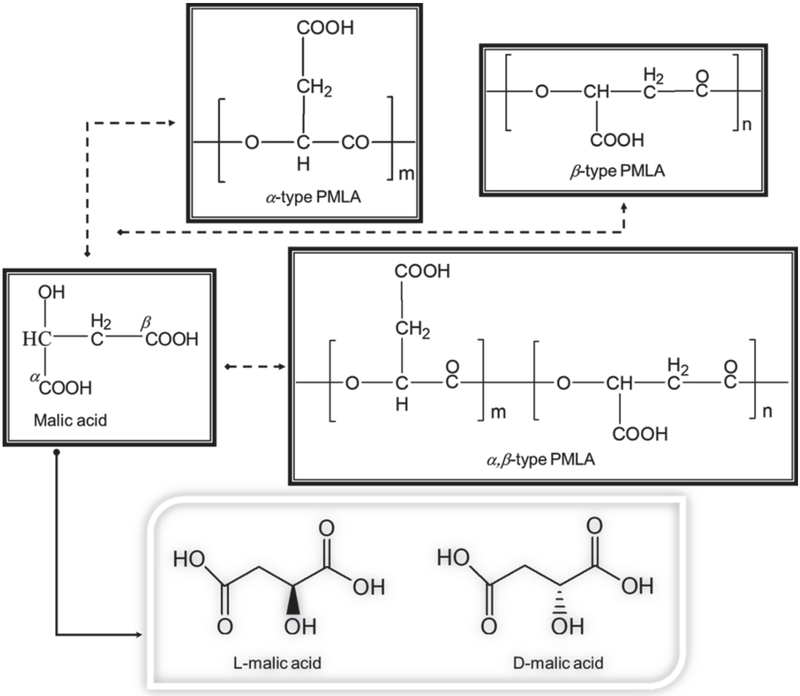

Figure 1-3. The molecular structures of α-, β-, and α,β-types of polymalate [83]. Figure was taken with permission from [83].

1.5 Potential applications of PMA

PMA stands out among biopolymers for its unique properties, including water solubility, biocompatibility, and ease of chemical modification due to abundant carboxyl groups [81, 90]. These features have positioned PMA as a promising material across a variety of applications. The following sections discuss PMA's primary biomedical applications, followed by other potential uses.

1.5.1 Biomedical applications

Compared to aliphatic biopolymers such as polyglycolic acid (PGA), polylactic acid (PLA), polycaprolactone (PCL), and poly lactic-co-glycolic acid (PLGA), PMA is water-soluble and it is chemically modifiable due to its abundant versatile pendant carboxyl groups [132, 133]. Moreover, the hydrolysis product of PMA is L-malate, which is non-toxic and non-immunogenic to human beings. Therefore, PMA represents a promising material for many pharmaceutical and biomedical applications.

Polycefin, a prototype of PMA-based nanoconjugate, was developed for the targeted carrier of monoclonal antibodies and antisense oligonucleotides to brain tumors and contains several modules chemically conjugated to PMA [84]. In addition, the polycefin nanoplatform has been used as a multifunctional delivery system across the blood-brain tumor barrier or blood-brain barrier for intra-brain targeting treatment [134, 135]. Another PMA-based nanoconjugate harboring glioblastoma tumor-targeting antibody (chimeric anti-human TfR) and blood-brain barrier targeting antibody (anti-murine TfR) displays a specific targeting to glioblastoma multiforme [136]. The targeted nanoscale immunoconjugates based on PMA that covalently linked checkpoint inhibitor antibodies to cytotoxic T-lymphocytea-associated antigen 4 and programmed cell death-1 were synthesized. It can deliver across the blood-brain barrier and trigger local brain anti-humor immune response [137].

A novel type of bifunctional nanoparticles fabricated by coating Fe_3O_4 nanoparticles with poly(DL-lactic aicd-co-α,β-malic acid) copolymer covalently linked fluorescein isothiocyanate. These nanoparticles can be detected by magnetic resonance and fluorescence imaging of human mesenchymal stem cells [138]. The fluorescent nano-imaging agent was synthesized and it has a high specificity for U87MG glioma cells, which can lead to long-lasting and intensive tumor fluorescence [139].

Recently, calcium PMA, a novel sustainable calcium supplement, can remarkably improve the conversion of serum calcium into bone calcium and promote osteogenesis and bone growth through *in vivo* mouse models. In addition, calcium PMA also stimulates osteoblast cell activity, mineralization, and proliferation [140]. Calcium PMA is advantageous against traditional calcium supplements due to its desirable water solubility [140]. Despite these advantages, calcium PMA has not been commercially available to date.

1.5.2 Other potential applications of PMA

PMA with high molecular weight (HMw) can be prospectively utilized as one constituent of coatings and films on vegetables, food, fruits, and pharmaceuticals [86, 141]. The covering of coatings and films is significant in preventing them from oxygen accession, moisture loss, pathogenic infection, and solute movement [141]. Recently, the PMA-based coating material containing 90 g/L HMw of PMA and 10% glycerol (g/100 g of HMw PMA) was first used in the protection of grapes. It was assessed that it can decrease weight loss, decay rate, respiration rate, relative conductivity, and vitamin C deprivation of grapes and it can enhance the texture including adhesiveness, gumminess, cohesiveness, hardness, and springiness. Meanwhile, the HMw PMA films can be easily degraded into malate in the mimic gastric fluid [142].

PMA consists of a large number of L-malate units, so the acid hydrolysis of PMA from large-scale fermentation can provide an alternative method for L-malate production [143]. It has been reported that malic acid can be used as an acidulant enhancer, metal cleaning, and textile finishing. Moreover, L-malate is an important C4 building block for the synthesis of other chemicals in the post-fossil fuel era [91, 143-146]. It was listed as one of the 12 most significant platform chemicals by the US Department of Energy in 2004 [147]. PMA can be utilized as an environmentally friendly chelating agent for heavy metal-contaminated soils, which is attributed to its multiple free carboxyl groups in the side chain [148]. It had a comparable chelating efficiency on cadmium compared to EDTA [149]. It was proposed that PMA can be used as second-generation sustainable bioplastics [87, 131]. A water-insoluble intelligent biomaterial can be designed based on a PMA scaffold. These can be used to fabricate a variety of solid devices such as microparticles, compression molded pellets, among others [150].

1.6 Synthetic routes of PMA

PMA production has primarily relied on two synthesis approaches: chemical and microbial. Chemical synthesis offers controlled polymerization techniques, while microbial synthesis uses natural pathways in specific microorganisms for efficient, renewable PMA production. Each method has distinct benefits and limitations concerning molecular weight, yield, scalability, and environmental impact. The following sections discuss these synthesis routes in detail, focusing on their processes, challenges, and applications

1.6.1 Chemical synthesis of PMA

As mentioned above, two types of chemical synthesis were reported: direct polycondensation and ring-opening polymerization [83]. L-malate can be directly polymerized into α,β-PMA under the catalysis of tin (II) chloride, tin (II) oxide, and tin (II) acetate through a one-step reaction [151]. The temperature of 110 °C is the most optimal to achieve 5,300 Da molecular weight of α,β-PMA by direct polycondensation using tin (II) chloride as a catalyst within 45 h