Biosynthesis of polymalic acid in fermentation: advances and prospects for industrial application

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ABSTRACT
Some microorganisms naturally produce β-poly(L-malic acid) (PMA), which has excellent water solubility, biodegradability, and biocompatibility properties. PMA has broad prospective applications as novel biopolymeric materials and carriers in the drug, food, and biomedical fields. Malic acid, a four-carbon dicarboxylic acid, is widely used in foods and pharmaceuticals, as a platform chemical. Currently, malic acid produced through chemical synthesis and is available as a racemic mixture of L- and D-forms. The D-form malic acid exhibits safety concerns for human consumption. There is extensive interest to develop economical bioprocesses for L-malic acid and PMA production from renewable biomass feedstocks. In this review, we focus on PMA biosynthesis by Aureobasidium pullulans, a black yeast with a large genome containing genes encoding many hydrolases capable of degrading various plant materials. The metabolic and regulatory pathways for PMA biosynthesis, metabolic engineering strategies for strain development, process factors affecting fermentation kinetics and PMA production, and downstream processing for PMA recovery and purification are discussed. Prospects of microbial PMA and malic acid production are also considered.

Introduction
β-Poly(L-malic acid) (PMA) discovery was first reported in 1969 and later isolated from the culture broth of Physarum polycephalum [1–3]. Biologically, PMA was found to bind DNA polymerases and act as a coordinator during DNA replication [4,5]. PMA, with its monomer L-malic acid being an intermediate metabolite in the tricarboxylic acid (TCA) cycle, can be decomposed biologically and is nontoxic to cells. PMA with many free carboxyl groups can be easily modified with other functional groups to generate novel PMA derivatives and to bind additional functional groups and small molecules for uses as carriers for drug delivery and as carrier-linked prodrugs [6,7]. With extensive water solubility, biodegradability and biocompatibility, PMA, as a novel biopolymer, has attracted increasing attention for its broad prospective applications in food, drug, biomedical, detergent, and environmental fields [1,8,9].

PMA is polymerized with L-malic acid (2-hydroxybutanedioic acid) as the sole monomer linked by ester bonds formed between –OH and α- or β-COOH. There are three configurations of PMA: α-type, β-type, and γ-type (see supplemental Figure S1). Among them, poly(β-L-malic acid) is the primary type found in microorganisms [10]. Several chemical synthesis routes involving complex reactions are available for PMA production from malic acid, which produces all three types of PMA with a relatively low-molecular weight [11–13] and are costly and difficult to scale up for commercial applications. PMA can also be produced from sugar by microorganisms, such as Aureobasidium pullulans, in fermentation, which is environmentally friendly and cost effective. Only β-type PMA is produced during fermentation, which has significant chemical and optical purity, high molecular weight, and excellent properties suitable for many applications [14].

The fermentation-produced PMA can be thermally hydrolyzed to L-malic acid, a flavor enhancer and acidulant widely used in the food, beverage, and cosmetic industries [15]. The application of malic acid in foods and pharmaceuticals is growing approximately 4%
 annually. As a dicarboxylic acid, malic acid is a promising platform chemical and a chemical building block for various high-volume products [16], with a predicted annual market of >200,000 tons [17]. Currently, malic acid is produced as a racemic mixture of D- and L-forms through the hydration of maleic acid chemically synthesized from petroleum-derived feedstocks. It is desirable to produce bio-based L-malic acid, which is not currently available commercially because of the high downstream processing costs to separate and purify malic acid from other organic acids, including succinate and acetate, which are also produced during fermentation. In contrast, PMA, as a polymer, can be easily separated from organic acids and other small molecules by ultrafiltration and alcohol precipitation [18], allowing its separation and purification in a simpler downstream process. PMA fermentation can thus provide an attractive biological route for manufacturing bio-based L-malic acid [19].

This review article provides an overview and prospects on microbial PMA production, including metabolic and regulatory pathways involved in PMA biosynthesis, metabolic engineering for strain development, fermentation kinetics and process optimization, and downstream processes for PMA separation and purification.

**PMA-producing microorganisms**

**Physarum polycephalum**

Shimada et al. [20] first reported that a polymer with L-malic acid as the structural unit inhibited the acid protease of *Penicillium cyclopium*. The polymer was later confirmed to be PMA. More recently, PMA was isolated from *Physarum polycephalum* and found to inhibit DNA polymerase α [21]. *P. polycephalum* belongs to *Myxomycota*, whose life cycle includes microscopic uninucleated amebae and multi-nucleated plasmodia stages. Only in the multi-nucleated plasmodia, PMA could bind and transport nucleic acid-related proteins in cells and temporarily inhibit the activities of some enzymes and structural proteins [22]. However, recent studies on PMA fermentation have focused on the yeast *Aureobasidium pullulans*, which can produce much more PMA than *P. polycephalum* can.

**Aureobasidium pullulans**

Nagata et al. [23] first discovered that the black yeast *A. pullulans* could produce PMA with mannitol as the main carbon source. Since then, many *A. pullulans* strains capable of producing PMA from glucose have been isolated [14,24]. Compared to *P. polycephalum*, *A. pullulans* is much better synthesizing and secreting of PMA with a molecular weight of 3–200 kDa. During fermentation with glucose as the substrate, *A. pullulans* can produce PMA at a high titer (up to 124 g/L), productivity (up to 1.22 g/L-h), and yield (up to 0.87 g/g) (see Table 1) suitable for industrialization.

*A. pullulans*, which belongs to the order Dothideales in the family Dothideaceae, is a polymorphic fungus widely found in terrestrial, aquatic, temporal, and polar areas. Its life cycle includes yeast-like, filamentous, chlamydospore-like, conidia, and various other morphologies [25]. Extracellular pH is a key factor regulating cell morphology transition from yeast-like to swollen cells [26]. Its phenotypic changes in colony morphology and physiology are also dependent on carbon and nitrogen sources, culture temperature, and colony age [27]. *A. pullulans* is polylextremotolerant that can adapt and survive in cold, hyper-saline, acidic, basic, and oligotrophic environments [27]. Its morphology transition is spontaneous behavior for alleviating environmental stress, but the role of rapid dimorphic switching during metabolite biosynthesis is not fully understood.

The genome of five different strains of *A. pullulans* has, to date, been fully sequenced [28]. All these strains have a large genome of ~30 Mb containing ~50% more genes than the genome of *Saccharomyces cerevisiae* (12.1 Mb, 6604 genes). Although there are significant differences in the genome assembly and annotation statistics of these *A. pullulans* strains (see supplemental Table S1), they all contain a large number of genes encoding secreted proteins that are associated with carbohydrate catabolism, including 180–267 glycoside hydrolases, 35–105 carbohydrate esterases, 10–15 glycosyltransferases, 4–11 polysaccharide lyases, and 44–59 carbohydrate-binding modules [28]. *A. pullulans* also has a large number (73–92) of sugar-transport proteins in the major facilitator superfamily (MFS), of which more than 75% are sugar: H⁺ symporters for various sugars, including sucrose, maltose, lactose, galactose, fructose, and xylose. These sugar: H⁺ symporters can operate either as uniporters or cation symporters. In contrast, *S. cerevisiae* has only 31 sugar-transport proteins and more than half are uniporters.

Besides PMA, *A. pullulans* can also produce pullulan, melanin, heavy oils (e.g. liamocins), antimicrobial compounds (including aureobasidin A, siderophores, and exophilin A), and various enzymes (e.g. amylases, β-fructofuranosidase, lipases, mannase, alkaline protease,
cellulases, and xylanase) [29,30], but many genes, associated with the biosyntheses of these metabolites, have not been identified or characterized, hindering their biotechnological applications.

Metabolic and regulatory pathways for PMA biosynthesis

Sugar metabolism and glucose repression

*A. pullulans* has ~800 secreted proteins, ~45% of them are related to enzymes active in degrading or catabolizing carbohydrates [28]. The genome of *A. pullulans* contains genes in most of the enzyme families involved in saccharide hydrolysis and sugar catabolism. The high number of sugar transporters and glycoside hydrolases affords the nutritional diversity of *A. pullulans*, allowing its use of various carbohydrates for growth and energy metabolism. As illustrated in Figure 1(A), all of the saccharides (stachyose, raffinose, sucrose, fructose, glucose, galactose, arabinose, and xylose) present in soy molasses can be used by *A. pullulans* ZX-10 for PMA production [19]. However, sucrose utilization has been found to be relatively slow, probably due to inhibition by glucose-mediated carbon catabolite repression (CCR) [31]. In the fermentation with sucrose as the substrate, it was rapidly hydrolyzed by secreted invertases into glucose and fructose which were uptaken by cells. However, fructose utilization was inhibited by glucose [32]. In general, glucose is the preferred carbon and energy source, and its presence may down-regulate transport and catabolism of other carbon sources by activating a complex regulatory network [33]. However, details of the signaling and regulatory pathways for sugar catabolism in *A. pullulans* have not been reported.

Biosyntheses of malic acid and PMA

Naturally, PMA is synthesized from L(+)-malic acid, an intermediate metabolite in the TCA cycle. Despite extensive strain screening and process optimization for PMA production, the PMA biosynthesis pathway in *A. pullulans* has not been fully elucidated. Figure 1(B) shows possible metabolic pathways with genes in *A. pullulans* leading to PMA, pullulan, and acetic acid biosyntheses. Possible mechanisms for PMA biosynthesis have been studied and proposed. Complete retention of the radioactivity in the PMA secreted by *P. polycephalum* cultivated on 14C-labeled glucose

| Strain | Substrate | Operating mode | Titer (g/L) | Mol mass (kDa) | Yield (g/g) | Productivity (g/L-h) | Reference |
|--------|-----------|----------------|-------------|----------------|-------------|-----------------------|-----------|
| A. pullulans ZD-3d | Glucose | Batch | 57.2 | 4.25 | 0.47 | 0.35 | [77] |
| Ipe-1 | Repeated batch | 57.4 | 19.2 | 0.27 | 1.3 | [85] |
| Sp. P6 | Batch | 91.1 | 81.37 | 0.67 | 0.52 | [93] |
| MCW | Batch | 117.4 | 205.4 | 0.87 | 1.22 | [94] |
| ZX-10 | Batch | 41.2 | – | 0.41 | 0.43 | [18] |
| | Fed-batch | 76.2 | – | 0.43 | 0.53 | |
| | FBB | 123.7 | – | 0.48 | 0.64 | |
| Sucrose | Batch | 48.5 | – | 0.68 | 0.26 | [32] |
| Sugarcane juice | Batch | 52.6 | – | 0.54 | 0.28 | |
| Soy molasses | Fed-batch | 116.3 | – | 0.41 | 0.43 | |
| Soybean hull | Fed-batch | 62.6 | – | 0.60 | 0.25 | [19] |
| | Fed-batch | 27.2 | – | 0.37 | 0.42 | |
| CCTC M2012223 | Sweet potato | Batch | 29.6 | – | 0.28 | 0.24 | [42] |
| | Fed-batch | 44.0 | – | 0.19 | 0.28 | |
| | FBB | 57.5 | – | 0.20 | 0.36 | |
| FJ-PYC | Sugarcane molasses | Batch | 81.5 | – | 0.62 | 0.67 | [64] |
| JY-MLS | Glucose + ethanol | Batch | 40.0 | – | 0.48 | 0.56 | [65] |
| HA-4D | Cane molasses | Fed-batch | 95.4 | – | 0.24 | 0.57 | [83] |
| | Jerusalem artichoke | Batch | 114.4 | – | 0.74 | 0.68 | [95] |
| | Batch | 10.1 | – | 0.30 | 0.40 | [82] |
| | Wheat straw | 23.5 | – | 0.30 | 0.40 | |
| JY6-11 | Glucose | Shake flask | 26.7 | 7.47 | – | 0.28 | [97] |
| | Xylose | Shake flask | 27.8 | 7.77 | – | 0.29 | |
| | Batch | 32.1 | – | 0.44 | 0.43 | |
| | Fed-batch | 80.4 | – | 0.47 | 0.52 | |
| | Corn cob | Batch | 28.6 | – | 0.37 | 0.40 | |
| var. melanogenum GXZ-6 | Sucre + citrate, malate | Batch | 62.6 | 2.797 | 0.46 | 0.35 | [98] |
| P. polycephalum ATCC204388 | Glucose | Batch | 2.7 | – | 0.06 | 0.02 | [36] |

FBB: fibrous bed bioreactor; SSF: solid state fermentation. 
*Converted from data reported for Ca2+ PMA with the conversion factor of 0.77.
bgds: gram dry substrate.
indicated that PMA was synthesized from glucose and its pathway should be closely related to the TCA cycle. Later, it was confirmed that the TCA cycle played an important role in PMA biosynthesis because trifluoroacetic acid (TFA) inhibited PMA production [34]. 14C-labeled malate microinjected into cells was found to be incorporated into PMA, confirming that malic acid was the immediate precursor of PMA biosynthesis [35]. CaCO3 in the fermentation medium was found to support PMA production in *P. polycephalam* [36]. A more recent study also showed that CaCO3 increased PMA production in *A. pullulans* [37]. 13C-labeled glucose and 13CO2 from Ca13CO3 were incorporated into malic acid and PMA in the presence of CaCO3, while malic acid and PMA were produced mainly from the oxidative reactions of TCA cycle and glyoxylate shunt when no CaCO3 was added in the medium [38].

Based on the aforementioned studies, we can conclude that the biosynthesis of malic acid in *A. pullulans* involves three pathways: (1) the oxidative TCA cycle in the mitochondria; (2) the reductive TCA (rTCA) pathway in the cytoplasm, where pyruvate carboxylase (PYC) converts pyruvate to oxaloacetate (OAA) and malate dehydrogenase (MDH) then converts OAA to malate; and (3) the glyoxylate pathway, in which OAA supplied via pyruvate carboxylation is converted to citrate (with the addition of acetyl-CoA) via citrate synthase, isocitrate lyase and finally malate (with the addition of acetyl-CoA) via malate synthase (MLS). The malate in the mitochondria is transported to the cytoplasm via a mitochondrial dicarboxylic acid transporter (DAT). The rTCA pathway is ATP neutral, involves CO2 fixation, and has the highest theoretical yield of 1.49 g/g or 2 mol/mol.
glucose, whereas the oxidative TCA cycle has the lowest theoretical yield of 1 mol/mol or 0.75 g/g glucose. The glyoxylate pathway has a theoretical yield of 1.33 mol/mol or 1.0 g/g glucose. Recently, a genome-scale metabolite model of *A. pullulans* CCTCC M2012223 (iZX637) was reconstructed and used for *in silico* analysis, showing that, with sucrose as the substrate, the majority of the substrate carbon flux goes through pyruvate, OAA (with CO₂ fixation), and then malate in the cytosol [39]. This genome-scale model confirms that the rTCA pathway is the most desirable or main pathway for high-yield malic acid (and PMA) biosynthesis in the presence of CO₂ in *A. pullulans*.

The key biosynthesis steps from malic acid to PMA have not been fully elucidated. It has been proposed that malate in the cytoplasm is polymerized into PMA, hypothetically with malyl-CoA as the intermediate, by malate-CoA ligase (MCL) and PMA synthase (PMS) [25,35]. However, little is known about PMS and the gene encoding this enzyme has not been identified or annotated in the sequenced genome. Since PMA has a similar ester bond structure to that of polyhydroxyalkanoate (PHA), which catalyzes the formation of poly(lactide), it is likely that PMS, if it exists, would have an analogous protein structure to that of PHA synthase (PhaC), which catalyzes the formation of polythioesters or PHA. Also, PMA biosynthesis could be via L-malyl-AMP involving malyl-AMP ligase and malyltransferase in an enzyme complex analogous to a non-ribosomal peptide synthetase (NRPS) [14]. DNA encoding the NRPS was found in the sequenced genome of *A. pullulans*. Further study is required to verify the genes and enzymes involved in the polymerization of malic acid to PMA.

The theoretical yield for PMA would be ~87% of that for malate due to the loss of 1 mol water for each mol malate polymerized. However, actual malate and PMA yields in fermentation were much lower than their theoretical values (see Table 1) because cell biomass and other products such as pullulan and acetate were also produced during the fermentation.

**Nitrogen stress responses and regulation of cell growth and PMA biosynthesis**

The amount and type of nitrogen source used in fermentation would affect not only cell growth but also PMA biosynthesis [41,42]. The nitrogen source or nutrient containing nitrogen not only provides the nitrogen needed for cell growth, but may also regulate metabolism and the synthesis of key metabolites [33]. A recent study showed that the medium containing 2 g/L NH₄NO₃ as the nitrogen source resulted in the highest PMA production from glucose by *A. pullulans*, while more NH₄NO₃ (10 g/L) increased cell growth but PMA production was significantly lower [43]. Furthermore, a lower nitrogen content (0.1 g/L NH₄NO₃) increased PMA product yield but limited cell growth and productivity.

The TOR (target of rapamycin) signaling pathway played an important role to regulate cell growth and metabolism in *A. pullulans* [43]. Both cell growth and PMA production decreased with increasing the rapamycin concentration in culture media, which corroborated well with the observed down-regulation of TOR1, DAT, and MCL by rapamycin [43]. In eukaryotes, nitrogen catabolite repression (NCR) is mediated via TOR kinase and its downstream signaling pathway [33]. TOR protein, a highly conserved Ser/Thr kinase found in all eukaryotic cells, is a global regulator of cell growth and metabolism. It functions by sensing amino acids and growth factors and integrating signals arising from the energy status and cellular stresses [44,45]. In *S. cerevisiae*, TOR proteins play key roles in nutrient-mediated signal transduction and cell growth control [46]. In response to rapamycin, stress, nutrient (amino acids), and hormone signals, TOR complex I (TORC1) regulates the phosphorylation of various proteins to control the expression of genes involved in ribosome biogenesis and a variety of metabolic pathways [47]. TORC1 responds to the nitrogen source via two major effector branches: Tap42–PPase complex and AGC kinase Sch9 (Figure 2). Sch9 is directly phosphorylated by TORC1 and regulates ribosome biogenesis at the transcriptional level, which also modulates cell growth. Tap42–PPase complex, which resides mainly in membranes, interacts with TORC1 and is released into the cytosol during nitrogen starvation, concomitant with the dephosphorylation of Tap42, which induces genes involved in nitrogen catabolism, the glycolytic pathway, and the TCA cycle [33]. In the presence of preferred or sufficient nitrogen sources, the activity of TORC1 and cell growth are sustained via a mechanism dependent on glutamine accumulation [48], which causes NCR or represses the genes involved in the catabolism of less preferred nitrogen sources by preventing nuclear localization of transcription factors Rtg1/3, Gln3, and Gat1 via their hyper-phosphorylation.

Most of the genes encoding enzymes and proteins in the TOR signaling pathway in *S. cerevisiae* are also present in the genome of *A. pullulans* CCTCC M2012223. A recent study, with comparative transcriptomics and proteomics profiling, showed that nitrogen availability significantly affected up- or down-regulation of many genes and proteins during energy, carbohydrate, amino acid, and lipid metabolism in *A. pullulans*.
CCTCC M2012223 [43]. Under nitrogen stress, several key genes in the TOR signaling pathway (tor1, tap42, and gat1) and PMA biosynthesis pathway (dat, mcl, and glk (glucose kinase)) were up-regulated, confirming that cells responded to nitrogen stress and regulated cell growth and PMA biosynthesis through the TOR signaling pathway.

Metabolic engineering for malate and PMA biosyntheses

Plasmids construction and transformation

Metabolic engineering of A. pullulans is limited by the available molecular biology tools. Several recombinant plasmids with antibiotic resistance and/or color selection markers, such as hyg (hygromycin B phosphotransferase), bar (phosphinothricin acetyltransferase), and gus (glucuronidase) have been developed and used for gene disruption and expression in A. pullulans [49–51]. Transformation by conventional electroporation or using PEG-mediated Li-acetate buffer did not work for A. pullulans. However, successful gene knockout and overexpression to enhance siderophore or pullulan biosynthesis in A. pullulans have been reported using spheroplasts for transformation [49–52]. More recently, Agrobacterium tumefaciens-mediated transformation (ATMT), which has been used in fungal genetic engineering [53], was developed for A. pullulans with good transformation efficiency. Since most plasmids transformed via ATMT would contain a single copy of T-DNA (transferred DNA), a random insertional mutant library would be generated after transformation [54]. This T-DNA-based random insertional mutagenesis can generate a large pool of mutants, which, however, would require an efficient screening method to find useful mutants for PMA production [55]. The T-DNA-based genome insertion can also be used for gene knock-in for stable gene expression in A. pullulans. More recently, several linear DNA fragments were simultaneously transformed by electroporation and then self-assembled and integrated via homologous recombination into the genome, resulting in gene knock-in or knock-out, in A. pullulans [56].

Overexpressing genes in the rTCA pathway and glyoxylate shunt

Rational metabolic engineering of A. pullulans to enhance its malate and PMA production has not been
reported to date. However, metabolic engineering has been successfully used to engineer various microorganisms for malate biosynthesis. As can be seen in the pathways leading to malic acid (Figure 3), the conversion of pyruvate to OAA by PYC would be an important controlling step for malic acid biosynthesis. A recent study showed that expressing an exogenous PYC in *Thermobifida fusca* increased its malate production by 47.9% [57]. Overexpressing indigenous PYC and MDH in the rTCA pathway and DAT (a C4-dicarboxylate transporter) resulted in more than a twofold increase in malate production from glucose by *Aspergillus oryzae*, reaching 154 g/L with high productivity (0.94 g/L-h) and yield (1.03 g/g) [58]. Expressing an exogenous PPC (phosphoenolpyruvate carboxylase) catalyzing the carboxylation of PEP to OAA, L-malate permease, and 6-phosphofructokinase encoded by pfk further improved malate production to 165 g/L with a productivity of 1.38 g/L-h in fed-batch fermentation [59]. Similarly, *Saccharomyces cerevisiae* was engineered to overexpress native PYC and MDH. A malate transporter from *Schizosaccharomyces pombe*, and the engineered strain was able to produce 59 g/L malate with a productivity of 0.19 g/L-h and yield of 0.31 g/g glucose [60]. In addition, *E. coli* has been engineered to redirect carbon flow for malate production by deleting multiple genes in its central anaerobic fermentation pathway and mutating fumarate reductase [61–63], and the best strain produced 69 g/L malate from glucose with a high yield of 1.04 g/g and productivity of 0.69 g/L-h [61].

These studies confirmed that the rTCA pathway is the most efficient one for malate biosynthesis. Since malic acid is the precursor (monomer) for PMA biosynthesis, one can expect that overexpressing genes encoding PYC, MDH, and DAT would also increase PMA production by *A. pullulans*. A recent study showed that overexpressing PYC resulted in a 15.1% increase in PMA production by *A. pullulans* compared to the wild-type strain [64]. Overexpressing MLS in the glyoxylate shunt also increased PMA production by 16.2% compared to the wild-type strain [65]. In addition, overexpressing *glk*, encoding glucose kinase, may also increase PMA production from glucose as suggested by a comparative transcriptomics analysis [43].

**Knockouts of genes in the pullulan biosynthesis pathway**

Pullulan, an extracellular high-molecular-weight polysaccharide composed of maltotriose units, can be produced from sugars including glucose, fructose, xylose, and sucrose at a high titer of 20–60.7 g/L and yield of 0.3–0.6 g/g sugar [66–68]. In our previous study, the strain ZX-10 mainly produced PMA (123.7 g/L at a yield of 0.55 g/g) with a modest level of pullulan (30 g/L at a yield of 0.12 g/g sugar) in glucose fermentation [18]. In addition, pullulan is also insoluble in alcohol [69] and may be co-precipitated with PMA by ethanol, requiring further purification to remove pullulans from PMA and rendering the downstream purification process more
complex. In addition, pullulan would increase the viscosity of fermentation broth, which may reduce mass transfer rates for oxygen and other nutrients, and thus lower cell productivity and product yield [70,71]. The pullulan biosynthesis pathway involves several enzymes, including α-phosphoglucose mutase, UDPG-pyrophosphorylase (UGPase), glucosyltransferases, and pullulans synthetase (see Figure 3). Although many genes involved in pullulan biosynthesis have not been elucidated, disrupting the pullulan synthetase gene (pull) in A. pullulans was effective in significantly reducing pullulan production and shifting the metabolic flux toward β-glucan or siderophore production [51,72]. However, the effect of pull disruption on PMA production has not been studied. Since overexpressing the endogenous UGPase gene increased pullulan production by A. pullulans [52], down-regulation of this gene may decrease pullulan and thus increase PMA production.

**Overexpression and knockout of genes in the TOR signaling pathway**

Genes involved in nitrogen metabolism and the TOR signaling pathway may also be good targets for metabolic engineering. As discussed earlier, under nitrogen stress the transcription factors Tap42 and Gat1 in the TOR signaling pathway were upregulated while PMA production also increased [43]. In addition, the AGC kinase Sch9, Rtg1/3 (see Figure 2) and glutamine synthetase, which catalyzes the amination of glutamate to glutamine and plays an essential role in nitrogen metabolism, would also be good targets for metabolic engineering, although their roles on regulating PMA biosynthesis in A. pullulans have not been studied.

**Fermentation process**

PMA fermentation is usually carried out in stirred-tank fermentors with pH, temperature and dissolved oxygen (DO) control. The optimal conditions for cell growth and PMA production were reported to be ∼25 °C, pH 6.0 and DO at 70% oxygen tension [18,73]. A higher temperature significantly decreased cell growth and PMA production. Little PMA was produced at 30 °C [73]. The fermentor is usually operated with vigorous agitation at 500–800 rpm and aeration at 1 vvm (vol. air per vol. liquid per min.), with DO controlled at 70%, which gave maximum PMA production [73]. A. pullulans cells are relatively hardy and not damaged by shear stress. A high agitation rate would promote cell division and growth. However, too much cell biomass production would reduce PMA yield. Lowering the agitation rate to 400 rpm after the initial growth phase decreased cell growth while maintaining DO at 70% improved PMA yield [70]. A DO of >50% also favored pullulan production [74], which not only reduced PMA yield but also increased broth viscosity and reduced oxygen transfer efficiency [75]. While a more intense aeration rate (up to 2 vvm) could increase oxygen transfer, which may lead to increased pullulan and reduced PMA production due to reduced CO2 availability for pyruvate carboxylation in the rTCA pathway. PMA productivity decreased when the culture redox potential increased from 57 to 100 mV in the late exponential growth phase due to the lack of reducing power, suggesting that the redox potential should be maintained below 70 mV to favor PMA biosynthesis [76]. In addition, several factors associated with the fermentation medium also affected PMA fermentation kinetics and overall process performance, which are discussed below.

**Carbon source**

Various simple sugars (glucose, fructose, xylose, galactose, maltose, sucrose, and lactose), soluble starch, glycerol, and several organic acids (sodium acetate, oxalic acid, sodium citrate, and sodium succinate) have been studied as carbon sources for PMA production under shake-flasks [73,77]. While A. pullulans could use all of the carbon sources studied, glucose and sucrose gave the highest PMA production, followed by xylose, fructose, and maltose. In addition, A. pullulans could also use oligosaccharides such as raffinose and stachyose [19]. However, polysaccharides, such as cellulose and xylan, and lipids have not been studied as a carbon source for PMA fermentation, although A. pullulans cultures were reported as excellent sources of cellulases, xylanase, and lipases [78–80]. While sodium acetate could not be utilized by A. pullulans, the addition of a small amount (∼5 g/L) of TCA cycle intermediates such as citrate, malate, fumarate, succinate, or OA were reported to increase PMA yield from glucose significantly [81]. However, one study showed that a supplementation amount of more than 5 g/L inhibited cell growth and PMA biosynthesis [77]. Furthermore, another study showed that adding TCA-cycle intermediates only increased cell growth without showing any significant effect on PMA production in fermentors under well-controlled pH and DO conditions, suggesting that addition of metabolic intermediates would not increase PMA production [73].

PMA production from low-cost substrates, including sugarcane juice, cane molasses, potato waste, soy molasses, Jerusalem artichoke tuber, corn cob, corn fiber, wheat straw, and soybean hull have also been
demonstrated. In general, yields and productivity from lignocellulosic biomass hydrolysates were lower than those from glucose (see Table 1), probably because of the inhibition caused by sugar and lignin degradation products (e.g. 5-hydroxymethylfurfural, furfural, acetic acid, and formic acid) [82]. Adaptation of cells to the hydrolysate could alleviate the inhibition by improving tolerance to toxic stress through, such as the activated sulfur assimilation pathway [82]. In addition, impurities present in crude substrates, such as heavy metals in cane molasses, might also inhibit cell growth and PMA production and must be removed or reduced to a nontoxic level [83]. Furthermore, PMA fermentation with crude substrates containing mixed sugars might be limited by glucose-mediated CCR as discussed earlier [32].

Nitrogen source

A. pullulans can utilize various nitrogen sources for growth. There was no significant difference in PMA production when different organic nitrogen sources, including yeast extract, tryptone, and corn steep liquor were tested [77], but NH4NO3 was found to be the best inorganic nitrogen source [82]. However, the amount of nitrogen source or the C/N ratio has a major impact on PMA production. For example, the medium containing 2 g/L NH4NO3 gave the highest PMA yield from glucose, whereas cell growth and fermentation were faster but PMA yield was lower at 10 g/L NH4NO3 [43]. Supplementing sugarcane juice or soy molasses with corn steep liquor reduced PMA yield due to too much of the nitrogen source promoting cell growth [19,32]. Similarly, higher production of pullulan by A. pullulans was also observed under nitrogen limitation, which affected the expression levels of many proteins associated with glycogen biosynthesis and transcriptional regulation [84].

CO2 fixation and carbonate addition

As discussed earlier, the rTCA pathway, which involves the carboxylation of pyruvate through CO2 fixation catalyzed by PYC, a biotin-containing enzyme, has the highest theoretical malic acid yield of 2 mol/mol glucose. Adding the cofactor biotin and extracellular CO2 donor, such as Na2CO3, were thus beneficial to PMA production [37]. To obtain a high PMA titer and yield, CaCO3 (30 g/L) is usually added in the fermentation medium not only to provide CO2 but also to buffer the pH at ~6.0 [18].

Neutralizing agent

Without the addition of a neutralizer during PMA fermentation, the medium pH would decrease quickly from ~6.0 to less than 4.0, which would strongly inhibit cell growth and PMA production. Consequently, it is thus necessary to maintain the medium pH around 6.0 by adding a neutralizing agent or base. CaCO3 is the most widely used neutralizing agent in PMA fermentation because it also provides exogenous CO2 for enhanced malic acid biosynthesis [18,36,77]. CaCO3 not only accelerated PMA production but also inhibited the accumulation of exopolysaccharide in A. pullulans ZD3d [77]. However, CaCO3 has a very low solubility in water (0.013 mg/mL at 20 °C), and its use as the neutralizing agent could produce large amounts of calcium residues in the fermentor. Other neutralizing agents such as NaOH, NH4OH, Na2CO3, and NaHCO3 have also been studied for PMA fermentation. Among them, Na2CO3 was reported to be the best one as it had a high solubility (29 g/100 mL at 20 °C) and could also provide CO2 to enhance PMA production [37]. While the addition of NH4OH increased cell growth, PMA production was reduced significantly due to the excessive nitrogen source promoting cell growth. A recent study found no difference in final PMA production titer by A. pullulans ipe-1 with NaOH or Na2CO3 as the neutralizing agent. However, adding 0.1 g/L CaCl2 increased PMA concentration by 11.4% [85]. PMA produced in batch fermentation with Na2CO3 as the neutralizing agent and CaCl2 addition also showed a significantly higher molecular weight (18.69 kDa vs. 12.52 kDa without CaCl2). The study suggested that Ca2+ might affect the yeast-like morphology favoring PMA production while CO32− had no effect. However, these studies used different strains, which might have contributed to different results. It is more likely that both CO32−, as a CO2 donor, and Ca2+, as a neutralizer and facilitator for membrane transport, are beneficial for PMA production.

Other factors

As already mentioned, the addition of biotin, the cofactor for PYC, was beneficial to PMA production [37]. In addition, Tween 80 (polyethylene glycol sorbitan monooleate) could also increase PMA production [41]. Tween 80, a widely used nonionic surfactant, not only enhanced oxygen uptake and CO2 evolution, it also dramatically changed cell morphology and upregulated mitochondrial dicarboxylate and transmembrane transporters [41]. Ethanol was also found to be an effective inducer that could regulate the expression level of MLS.
and strengthen the carbon flux through the glyoxylate shunt for PMA biosynthesis [65].

**Bioreactor operation mode**

The performance of PMA fermentation is also affected by the type and operation mode of the bioreactor. Submerged fermentation with free cells in a stirred-tank bioreactor is more widely used in PMA fermentation studies. In general, a higher final PMA titer can be obtained in fed-batch fermentation, while higher volumetric productivity is obtained in repeated batch fermentation with cell recycle due to significantly increased cell density (see Table 1). Fermentation carried out in a fibrous bed bioreactor (FBB) with immobilized cells also showed improved PMA production [18,42]. More recently, untreated agricultural residues were used to produce PMA in solid-state fermentation, which may offer an economically attractive way for PMA production from low-cost solid biomass feedstocks [86].

**Downstream process for PMA separation and purification**

PMA produced during fermentation was mainly secreted into the culture broth, which also contained small amounts of peptides/proteins, pigments, polysaccharides, organic acids, and various inorganic salts. These impurities need to be removed during downstream processing. PMA in both undissociated acid and dissociated anionic forms are highly soluble in water, but only the acid form can be dissolved in organic solvents such as acetone. A crude PMA product can be obtained through precipitation with an alcohol such as methanol or ethanol. The precipitation of PMA is affected by the PMA concentration, molecular weight, solution pH, and temperature. However, pullulan, which is usually also co-produced with PMA by *A. pullulans*, can also be precipitated by organic solvents. Since pullulan and PMA have different molecular weights and structures, they can be separated by fractional precipitation in organic solvents. For example, pullulan was selectively removed as a precipitate after adding 0.5 vol. of methanol to the fermentation broth, and Ca-PMA in the supernatant was then precipitated with 2 vol. of methanol at 4 °C for overnight [77]. Melanin pigment, if present, can be easily demelanized or decolored by oxidation with 4% (w/v) H₂O₂ [87].

Holler [88] developed a process for PMA recovery from the culture broth, starting with adsorption on DEAE-cellulose resins followed with washing and eluting with sodium phosphate buffer containing 0.2–1.5 M NaCl, precipitation with 70% (v/v) ethanol and 0.2 M NaCl, and then freeze-drying to concentrate the PMA. Salts were then removed by size-exclusion chromatography using Sephadex G25, and the PMA sodium salt was converted into the acid form over Amberlite 120H⁺. Followed with freeze-drying and dissolving in anhydrous acetone, a solid, purified, colorless PMA was produced, with an overall product yield of 20–30%. A simpler PMA purification process consisting of ethanol precipitation, adsorption with IRA-900 anion-exchange resins, and elution with NaCl solution, gave a ~100% pure PMA product with a high recovery yield of 84% [18]. PMA can be hydrolyzed to L-malic acid by heating or by adding sulfuric acid to break the ester bonds. It is noted that cells can be removed to generate a clear broth before further downstream processing [89]. Figure 3 illustrates the downstream process for PMA production involving membrane filtration for cell separation, alcohol precipitation for PMA recovery, decolorization, adsorption with IRA-900 resin, and drying to the final powder product. Cost analysis showed that PMA and malic acid could be produced at competitive prices from sugarcane juice and soy molasses using the processes discussed above [19,32].

**Conclusions and prospects**

*A. pullulans* has a large genome, can secrete a large number of hydrolases capable of degrading various plant materials, and possesses diverse metabolic and biosynthetic pathways. It can produce a wide variety of extracellular metabolites, including polymers such as PMA, from abundant, renewable, low-cost biomass feedstocks such as soy molasses [19]. PMA with unique properties different from those of more familiar and well-studied biopolymers, including poly-lactic acid (PLA), PHA, and poly-glutamic acid (PGA), has many potential applications as carriers for drugs and flavor compounds [90]. Its monomer (L-malic acid) is widely used as an acidulant and flavor enhancer in foods and is a platform chemical with a large market of 200,000 tons. However, current production of PMA and malic acid via chemical synthesis is not sustainable. Naturally, many microorganisms can produce malic acid as the main metabolite [15,91]. Several filamentous fungi, including *Penicillium viticola* 152 [92] and *Aspergillus oryzae* [58,59], are capable of producing malic acid at a high titer (>150 g/L), yield (~1.0 g/g), and productivity (>1 g/L·h). However, to date bioproduction of malic acid has not been commercialized with any of the malate-overproducing strains, both
native and engineered, largely because of the difficulty and high cost in separating and purifying malic acid from other organic acids (e.g. acetic, succinic, fumaric, and citric acids) co-produced in the fermentation [18]. Malic acid production via PMA, which can be easily concentrated via ultrafiltration followed with alcohol precipitation without contamination by other acids, offers an advantageous alternative to malate fermentation [18]. Compared to malic acid fermentation, PMA fermentation also requires 50% less neutralizer and is not inhibited by its product PMA. Unfortunately, current PMA fermentation has relatively low product yield and productivity, as compared to malic acid fermentation. In addition, pullulan is usually also produced as a byproduct, which not only lowers the PMA product yield but also makes product recovery and purification more difficult and costlier. It is thus desirable to further improve PMA fermentation via metabolic and process engineering.

The application of A. pullulans for industrial production of PMA and malic acid from low-cost renewable substrates requires a better understanding of A. pullulans in its carbon and nitrogen metabolism and regulation on cell growth and PMA biosynthesis. In particular, the TOR signaling pathway should be studied for its role in regulating nitrogen and carbon metabolisms in A. pullulans at the systems level via transcriptomics, proteomics, and metabolomics analyses. Such studies can provide the knowledge needed for metabolic engineering of this yeast. In addition, novel genome editing tools, such as CRISPR-Cas9, which has been widely applied to mammalian cells and many yeasts and bacteria, should also be developed and used for metabolic engineering of A. pullulans. Furthermore, enzymes such as PMS involved in the malic acid polymerization reactions for PMA production need to be identified and characterized, which may provide a novel biosynthesis pathway for producing biopolymers from other organic acids. An efficient PMA bioproduction process will benefit not only economic development but also the environment by providing green chemical products and reducing greenhouse gas emissions via CO₂ fixation in the fermentation process.

Disclosure statement
No potential conflict of interest was reported by the authors.

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