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Analysis of the L-malate biosynthesis pathway involved in poly(β-L-malic acid) production in *Aureobasidium melanogenum* GXZ-6 by addition of metabolic intermediates and inhibitors[§]

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Poly(β-L-malic acid) (PMA) is a promising polyester formed from L-malate in microbial cells. Malate biosynthesis is crucial for PMA production. Previous studies have shown that the non-oxidative pathway or oxidative pathway (TCA cycle) is the main biosynthetic pathway of malate in most of PMA-producing strains, while the glyoxylate cycle is only a supplementary pathway. In this study, we investigated the effect of exogenous metabolic intermediates and inhibitors of the malate biosynthetic pathway on PMA production by *Aureobasidium melanogenum* GXZ-6. The results showed that PMA production was stimulated by maleic acid (a fumarase inhibitor) and sodium malonate (a succinate dehydrogenase inhibitor) but inhibited by succinic acid and fumaric acid. This indicated that the TCA cycle might not be the only biosynthetic pathway of malate. In addition, the PMA titer increased by 18.1% upon the addition of glyoxylic acid after 72 h of fermentation, but the PMA titer decreased by 7.5% when itaconic acid (an isocitrate lyase inhibitor) was added, which indicated that malate for PMA production was synthesized significantly via the glyoxylate cycle rather than the TCA cycle. Furthermore, *in vitro* enzyme activities of the TCA and glyoxylate cycles suggested that the glyoxylate cycle significantly contributed to the PMA production, which is contradictory to what has been reported previously in other PMA-producing *A. pullulans*.

***Keywords*:** poly(β-L-malic acid), *Aureobasidium melanogenum*, biosynthetic pathway, glyoxylate cycle, TCA cycle

Introduction

Poly(β-L-malic acid) (PMA) is a microbial polyester that is formed by the interlinkage of L-malate molecules via ester bonds between the α-hydroxyl groups and β-carboxyl groups (Chi *et al.,* 2016a). PMA and its derivatives have a wide range of applications in medicine, food and other industries, for example, as drug carriers, surgical sutures, and biodegradable plastics, based on their excellent biochemical properties, such as water-solubility, biocompatibility, biodegradability, and chemical modifiability (Ding *et al.,* 2013).

PMA can be produced by several microorganisms, such as *Penicillium cyclopium* (Shimada *et al.,* 1969), *Physarum polycephalum* (Fischer *et al.,* 1989) and *Aureobasidium pullulans* (Nagata *et al.,* 1993). Among these strains, *A. pullulans* was confirmed to have the highest PMA synthesis activity. However, regardless of the microorganism used to produce PMA, L-malate is the only precursor (Holler *et al.,* 1992; Schmidt *et al.,* 1996). As shown in Fig. 1, there are three metabolic pathways for malate synthesis in microorganisms, namely, the non-oxidative and oxidative pathways (TCA cycle), and the glyoxylate cycle (Chi *et al.,* 2016b). Several studies have reported that the malate biosynthesis pathway for PMA production varies among strains. For example, Liu and Steinbüchel (1997) reported that both the TCA cycle and glyoxylate cycle were involved in PMA synthesis in *A. pullulans* CBS 591.75, based on the observation that trifluoroacetic acid inhibited, while malonate, maleate, succinate, and malate enhanced PMA production (Liu and Steinbüchel, 1997). However, Lee and Holler (2000) found that PMA synthesis in *P. polycephalum* was significantly associated with the TCA cycle because succinate and malate stimulated, while trifluoroacetic acid and malonate inhibited PMA production (Lee and Holler, 2000). Interestingly, Cao *et al.* (2014) reported that exogenous addition of trifluoroacetic acid, succinate and malate had negligible effects on the PMA production in *A. pullulans* ipe-1, indicating that the malate for PMA synthesis was most likely derived from a non-oxidative pathway (Cao *et al.,* 2014). All of the above hypotheses regarding the malate biosynthesis pathway involved in PMA production were based on the effects of exogenous metabolic intermediates and inhibitors on PMA production. However, further in-depth studies have not been conducted, such as investigations of changes in the activities of key enzymes and the expressions of the genes encoding the enzymes in these biosynthetic pathways.

In our previous study, the novel strain *Aureobasidium melanogenum* GXZ-6, with the ability to produce PMA was

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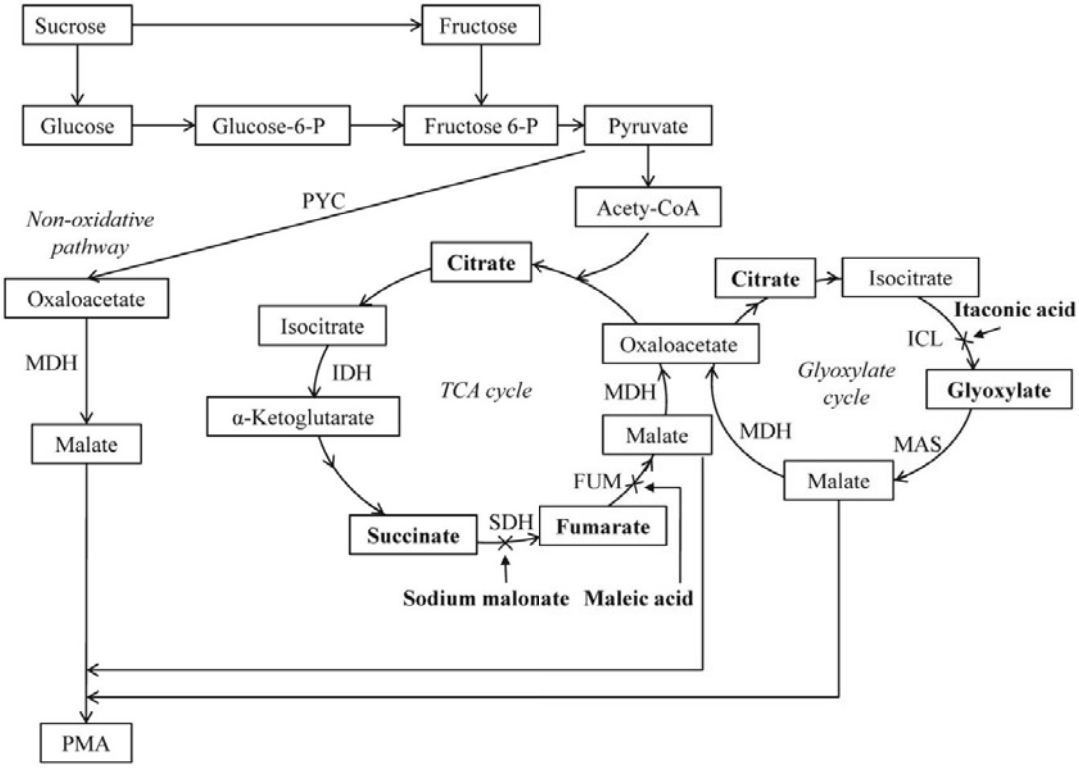

Fig. 1. Proposed PMA biosynthesis pathways in *A. pullulans*. PYC, pyruvate carboxylase; MDH, malate dehydrogenase; IDH, isocitrate dehydrogenase; SDH, succinate dehydrogenase; FUM, fumarase; ICL, isocitrate lyase; MAS, malate synthetase. The intermediates and inhibitors tested in this study are highlighted in bold.

isolated and characterized (Zeng *et al.,* 2018). In this study, the effects of exogenous metabolic intermediates and inhibitors of the malate biosynthetic pathway on PMA production were investigated, and the results showed that the malate for PMA synthesis in GXZ-6 might be derived significantly from the glyoxylate cycle, unlike other PMA-producing *A. pullulans*. Furthermore, the activities of key enzymes, transcription of the genes encoding the key enzymes and intracellular concentrations of key metabolites in the TCA and glyoxylate cycles were analyzed in detail.

Materials and Methods

Microorganism and medium

In this study, *A. melanogenum* GXZ-6 (CCTCC M 2017517) was used as a PMA producer. This strain was maintained on a potato dextrose agar slant and stored at 4°C. The seed medium was composed of 80 g/L sucrose, 2 g/L $NaNO_3$, 0.5 g/L KCl, 0.1 g/L K_2HPO_4, 0.1 g/L $MgSO_4{\cdot}7H_2O$, 0.05 g/L $ZnSO_4{\cdot}7H_2O$, and 20 g/L $CaCO_3$. The fermentation medium was composed of 160 g/L sucrose, 4 g/L $NaNO_3$, 0.5 g/L KCl, 0.1 g/L KH_2PO_4, 0.1 g/L $MgSO_4{\cdot}7H_20$, 0.2 g/L $ZnSO_4{\cdot}7H_2O$, and 40 g/L $CaCO_3$. The initial pH values of the seed medium and fermentation medium did not need adjustment.

Cultivation conditions

For the seed culture, a lawn of GXZ-6 grown on a potato dextrose agar slant was scraped into a 500-ml flask containing 100 ml of seed medium and then cultured with oscillatory at 30°C and 180 rpm for 36 h. For shake flask fermentation, 4 ml of seed culture was inoculated into 40 ml of fermentation medium in a 250-ml flask and fermented at 30°C for 8 days with shaking at 180 rpm. To investigate the effects of exogenous metabolic intermediates and inhibitors on the production of PMA, citric acid, succinic acid, fumaric acid, maleic acid, sodium malonate, glyoxylic acid and itaconic acid at a concentration of 5 g/L and biotin at a concentration of 5 mg/L were added to the fermentation medium at the beginning or after 72 h of fermentation.

Enzyme activity assay

GXZ-6 cells were harvested by centrifugation at 9,987 × *g* for 10 min and washed twice with sterilized physiological saline. Collected cells were resuspended in 5 ml of Tris/HCl buffer (1 M, pH 7.0) and sonicated at 400 W for 15 min by 2-sec working pulses with 3-sec cooling intervals. The cell debris was removed by centrifugation at 22,471 × *g* for 30 min, and the crude extracts were used for the enzyme activity assay. The above procedures were performed at 4°C. Enzyme activities, including isocitrate lyase (ICL), malate synthase (MAS), isocitrate dehydrogenase (IDH), and fumarase (FUM) activities were measured according to previously reported methods (Massey 1953; Giachetti *et al.,* 1988; Jong-Gubbels *et al.,* 1995; Yang *et al.,* 2002). Detailed procedures are provided in the Supplementary Material.

Real-time polymerase chain reaction

RNA extraction and the reverse transcriptase reaction were performed using the TRNzol universal reagent (Tiangen) and the HiScript II Q RT SuperMix (Vazyme), respectively. The quantitative real-time polymerase chain reaction (qPCR) for relative quantification was performed using the ChamQ Universal SYBR qPCR master mix (Vazyme) on a 7500 real-time PCR system (Applied Biosystems). The genes encoding ICL (*icl*), MAS (*mas*), IDH (*idh*), and FUM (*fum*) were analyzed by qPCR. The gene encoding actin, namely *act* (GenBank: EU855739), was selected as the endogenous reference gene. The primers used to amplify these genes were designed using Primer Premier 5.0 software, and are shown in Supplementary data Table S1. Comparative analysis by the $2^{-\Delta\Delta CT}$ method was used to calculate the fold changes in gene expression relative to the initial control (Livak and Schmittgen, 2001).

Intracellular metabolite analysis

The cell broth was quenched in precooled methanol (60%, v/v) with a volume ratio of 1 to 5. Then, the crude extracts for intracellular metabolite analysis were obtained under the same conditions as those used for the enzyme activity assay. Citric acid, succinic acid and fumaric acid levels were measured using a Shimadzu HPLC system equipped with a JADE-PAK carbohydrate H^+ column (7.8 mm × 300 mm; Techway) and a UV detector (210 nm). A total of 5 µl of sample was subjected to analysis at 50°C using H_2SO_4 (2.75 mM) as the eluent with a flow rate of 0.6 ml/min (Chinnici *et al.,* 2005). Malic acid and glyoxylic acid were measured using a Shimadzu HPLC system equipped with a JADE-PAK ODS-AQ C18 column (4.6 mm × 250 mm; Techway) and a UV detector (210 nm). A total of 5 µl of sample was subjected to analysis at 40°C using $NH_4H_2PO_4$ (2.5 mM, pH 2.5) as the eluent with a flow rate of 0.7 ml/min (Yang *et al.,* 2011).

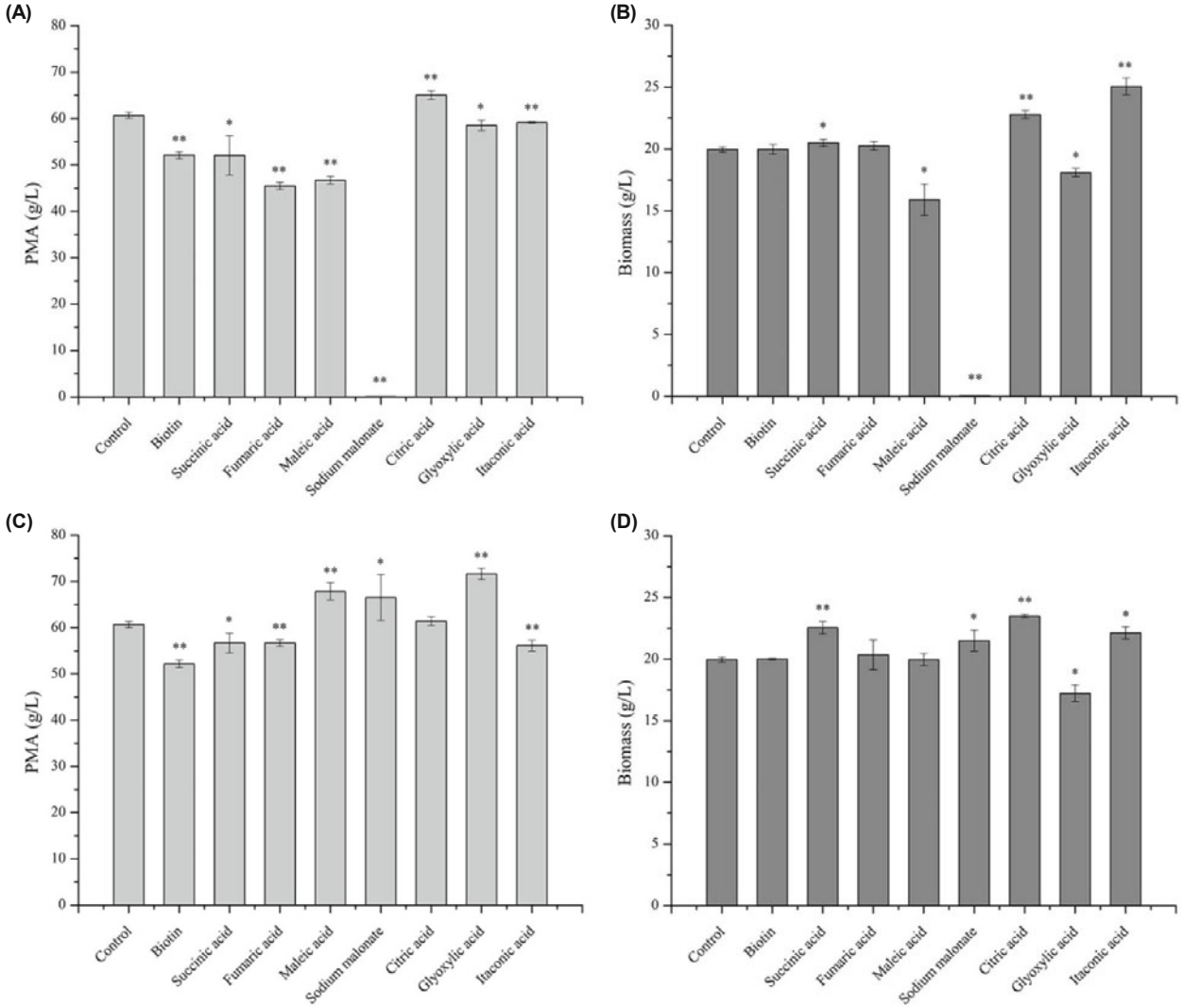

Fig. 2. Effects of exogenous metabolic intermediates and inhibitors on PMA production. Citric acid, succinic acid, fumaric acid, maleic acid, sodium malonate, glyoxylic acid, and itaconic acid at a concentration of 5 g/L and biotin at a concentration of 5 mg/L were added to the fermentation medium at the beginning (A and B) or after 72 h (C and D) of fermentation. The control experiment was conducted in the fermentation medium without any exogenous metabolic intermediates or inhibitors. Each experiment was performed in triplicate, and the error bars represent standard deviations. Asterisks indicate significant differences between the experimental group and the control group according to Student's *t*-test; * $P \leq 0.05$, ** $P \leq 0.01$.

Analytical methods

Dry cell weight (DCW) was measured according to a previously reported method (Zou *et al.,* 2016). In brief, 10 ml of fermentation broth was collected, and an appropriate amount of HCl (3 M) was added to eliminate $CaCO_3$. The broth was then centrifuged at 16,700 × *g* for 10 min. The sedimentary cells were obtained and washed twice with sterilized water and then dried at 105°C to a constant weight. The PMA concentration was analyzed by HPLC and calculated by measuring the difference between L-malate levels before and after hydrolysis of the fermentation broth. In brief, cells in fermentation broth were removed by centrifugation at 16,700 × *g* for 10 min, and then 3 ml of 2 M H_2SO_4 was added to 3 ml of the supernatant, and hydrolysis was performed at 90°C overnight (Nagata *et al.,* 1993). The hydrolysate was centrifuged at 16,700 × *g* for 5 min to remove insoluble contaminants and then neutralized by 2.5 M NaOH. The concentration of L-malate in the fermentation broth and hydrolysate was measured using a Shimadzu HPLC system equipped with a JADE-PAK ODS-AQ C18 column (4.6 mm × 250 mm; Techway) and UV detector (210 nm). A total of 5 µl of sample was subjected to analysis at 25°C using KH_2PO_4 (50 mM, pH 2.5) as the eluent with a flow rate of 0.7 ml/min.

Statistical analysis

Each experiment was performed in triplicate. All data are expressed as the means ± SDs. Student's *t*-test was used to interpret the differences among the data. Differences between groups were considered statistically significant for *P*-values ≤ 0.05 or *P*-values ≤ 0.01.

Results and Discussion

Effects of exogenous metabolic intermediates and inhibitors on PMA production

L-Malate is the only precursor of PMA, so the production of PMA is mainly associated with malate biosynthesis. As shown in Fig. 1, there are three metabolic pathways for malate synthesis in microorganisms (Chi *et al.,* 2016b). To identify the main metabolic pathway that provides malate for PMA production in *A. pullulans* GXZ-6, the effects of exogenous metabolic intermediates and inhibitors on the cell growth and PMA production were investigated (Fig. 2). These metabolites were added at the beginning or after 72 h of fermentation so that the effects on the cell growth and PMA production could be estimated separately.

In the non-oxidative pathway, pyruvate is carboxylated to oxaloacetate by a biotin-dependent pyruvate carboxylase and then reduced to malate by an NAD(H)-dependent malate dehydrogenase (Chi *et al.,* 2016b). As shown in Fig. 2, regardless of the time point at which biotin was added to the medium, the biomass exhibited no significant change, and the PMA titer decreased slightly. The results indicated that the non-oxidative pathway may not be involved in the biosynthesis of malate because the biotinylated enzyme, namely, pyruvate carboxylase (Wallace *et al.,* 1998), was not activated by biotin.

In the oxidative pathway, acetyl-coenzyme A and oxaloacetate are condensed to citrate, and then oxidized to malate via the TCA cycle (Chi *et al.,* 2016b). Succinic acid and fumaric acid are the metabolic intermediates of the TCA cycle. The results showed that the PMA titer decreased, but the biomass increased to varying degrees, regardless of the time point at which the two metabolic intermediates were added into the medium (Fig. 2). Maleic acid and sodium malonate are inhibitors of fumarase and succinate dehydrogenase (Liu and Steinbüchel, 1997), respectively. In particular, there was no cell growth and PMA production when sodium malonate was added into the medium at the beginning of fermentation. This effect may be caused by suppression of cell respiration by sodium malonate (Rikhvanov *et al.,* 2003). Then, the PMA titer increased by 11.8% and 9.7% when maleic acid and sodium malonate were added into the medium after 72 h of fermentation, respectively (Fig. 2C). If the synthesis of malate for PMA production is associated with the TCA cycle, the addition of either of these two inhibitors should decrease the PMA titer. Thus, the results suggested that the TCA cycle might not be the only biosynthetic pathway for malate but mainly supports cell growth as the TCA cycle can produce a large amount of ATP (Bott and Eikmanns, 2013). Moreover, the PMA titer and biomass decreased significantly, even exhibiting complete inhibition, as maleic acid and sodium malonate were added into the medium at the beginning of fermentation (Fig. 2A and B), which further indicated the importance of the TCA cycle for cell growth.

Citric acid is the initial metabolite of the TCA cycle. Interestingly, both the PMA titer and biomass increased when citric acid was added to the medium. Specifically, the PMA titer increased by 7.2% as citric acid was added into the medium at the beginning of fermentation (Fig. 2A). However, inhibition of fumarase and succinate dehydrogenase demonstrated that the TCA cycle might not be the only biosynthetic pathway for malate. This result indicates that there exists another pathway that provides malate for PMA production. The glyoxylate cycle is a supplementary pathway of the TCA cycle, converting isocitrate to malate via ICL and MAS (Chi *et al.,* 2016b). As shown in Fig. 2C, the PMA titer increased by 18.1% when glyoxylic acid was added into the medium after 72 h of fermentation, but the PMA titer decreased by 7.5% when itaconic acid (an ICL inhibitor) was added. Additionally, regardless of whether citric acid and itaconic acid were added simultaneously or citric acid was added before itaconic acid, the PMA titer was lower than that observed when only citric acid was added (Supplementary data Fig. S1). These results suggested that malate for PMA production was synthesized significantly via the glyoxylate cycle rather than the TCA cycle.

Analysis of key enzyme activities and transcription of the genes encoding these enzymes of the TCA cycle and glyoxylate cycle

To further study the effects of citric acid and sodium malonate on the biosynthesis of malate for PMA production, the activity of key enzymes (ICL, MAS, IDH, FUM) and the transcription of the genes encoding these enzymes were investigated. Among these enzymes, ICL and MAS are the key enzymes of the glyoxylate cycle, while IDH and FUM are the key enzymes of the TCA cycle. Citric acid was added at the

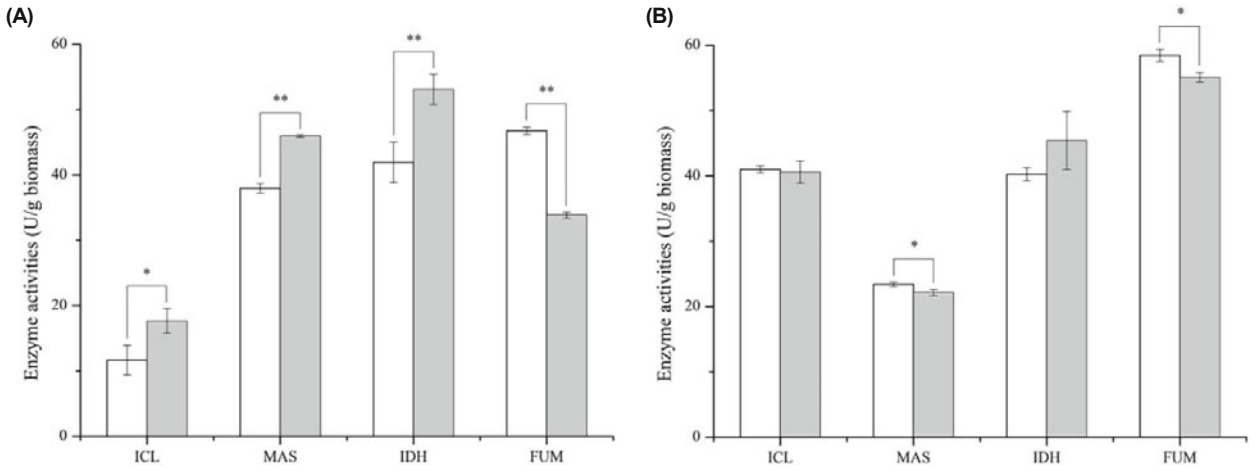

Fig. 3. Effects of citric acid and sodium malonate on the key enzyme activities in the TCA cycle and glyoxylate cycle. Figure 3A shows the data collected for GXZ-6 cells cultured for 48 h with the addition of citric acid at the beginning of fermentation (closed bars). Medium without citric acid was used as a control (open bars). Figure 3B shows the data collected for GXZ-6 cells cultured for 96 h with sodium malonate added after 72 h of fermentation (closed bars). Medium without sodium malonate was used as a control (open bars). ICL, isocitrate lyase; MAS, malate synthetase; IDH, isocitrate dehydrogenase; FUM, fumarase. Each experiment was performed in triplicate, and the error bars represent standard deviations. Asterisks indicate significant differences according to Student's *t*-test; * $P \leq 0.05$, ** $P \leq 0.01$.

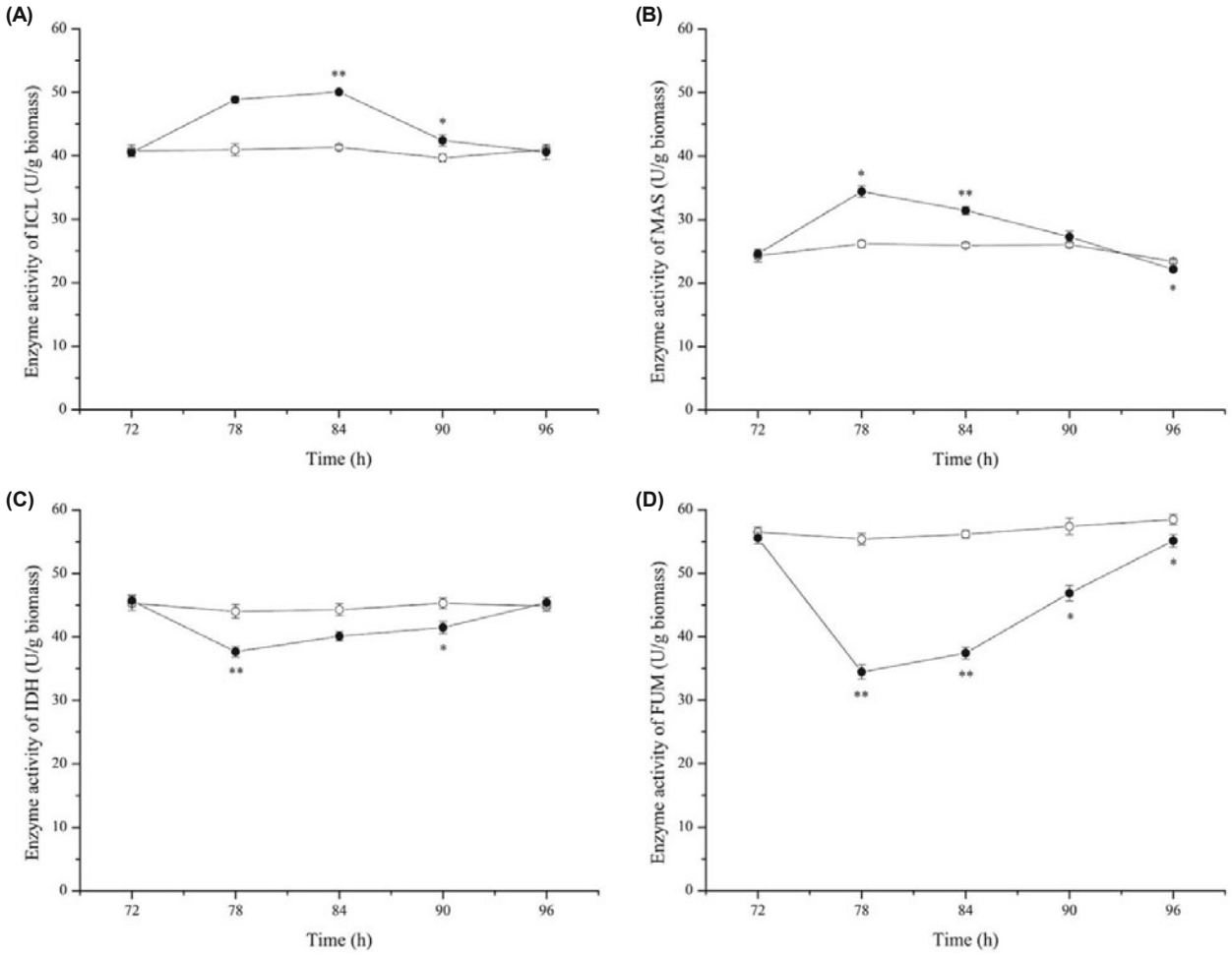

Fig. 4. Effects of sodium malonate on the key enzyme activities in the TCA cycle and glyoxylate cycle. The time courses shown are for ICL (A), MAS (B), IDH (C), and FUM (D). The cells were cultured as described in Fig. 3B in control (○) and sodium malonate-containing (●) media. The control experiment was conducted in fermentation medium without any exogenous metabolic intermediates or inhibitors. Each experiment was performed in triplicate, and the error bars represent standard deviations. Asterisks indicate significant differences according to Student's *t*-test; * $P \leq 0.05$, ** $P \leq 0.01$.

Table 1. Changes in PMA titer, biomass and intracellular organic acids levels with the addition of citric acid and sodium malonate in the fermentation medium of *A. melanogenum* GXZ-6

Item	PMA (g/L)	Biomass (g/L)	Citrate (mg/g biomass)	Succinate (mg/g biomass)	Fumarate (mg/g biomass)	Glyoxylate (mg/g biomass)	Malate (mg/g biomass)
Control[a]	11.7 ± 0.16	5.4 ± 0.13	2.93 ± 0.19	9.05 ± 0.13	0.12 ± 0.01	3.7 ± 0.13	3.23 ± 0.75
Add citric acid[b]	12.3 ± 0.15	6.8 ± 0.22	45.87 ± 0.32	8.31 ± 0.31	0.4 ± 0.02	4.04 ± 0.1	6.73 ± 0.22
Control[c]	26.3 ± 0.51	10.6 ± 0.12	1.53 ± 0.02	1.33 ± 0.18	0.04 ± 0.01	7.84 ± 0.85	2.63 ± 0.03
Add sodium malonate[d]	27.4 ± 0.36	10.4 ± 0.03	2.69 ± 0.15	2.49 ± 0.84	0.04 ± 0.04	6.09 ± 0.19	4.6 ± 0.51

[a] Data were collected from GXZ-6 cultured in fermentation medium for 48 h.
[b] Citric acid, used as metabolic intermediate, was added into the fermentation medium at the beginning of fermentation; GXZ-6 was cultured for 48 h; and the data were collected.
[c] Data were collected from GXZ-6 cultured in fermentation medium for 96 h.
[d] Sodium malonate, used as a metabolic inhibitor, was added into the fermentation medium after 72 h of fermentation; then GXZ-6 was cultured for 96 h; and the data were collected.

beginning of fermentation, and then, the activities of the key enzymes and the transcriptional levels of the genes encoding these enzymes were detected after 48 h of fermentation. The activities of ICL and IDH were increased considerably, indicating that the TCA cycle and glyoxylate cycle might be stimulated by citric acid (Fig. 3A). Interestingly, the transcriptional level of the *icl* gene increased 1.56-fold, but that of the *idh* gene exhibited little change (Supplementary data Fig. S2A). This finding indicated that the stimulatory effect of citric acid on the glyoxylate cycle may be stronger than that on the TCA cycle. In addition, the enzyme activity and gene transcriptional level of MAS increased, but those of FUM decreased. This finding further demonstrated that the glyoxylate cycle was more active than the TCA cycle.

Sodium malonate was added after 72 h of fermentation, and the activity of key enzymes and the transcriptional levels of the genes encoding these enzymes were detected after 96 h of fermentation. As shown in Fig. 3B and Supplementary data Fig. S2B, the activities of the four enzymes and the transcriptional levels of the genes encoding these enzymes did not change significantly, especially those of ICL and MAS (Fig. 3C and D). We hypothesized that this result might be due to sodium malonate being a structural analog to succinate and a competitive inhibitor of succinate dehydrogenase (Garraway and Evans, 1984). The inhibitory effect of sodium malonate on succinate dehydrogenase was eliminated 24 h after treatment with the compound. Thus, the activities of the four enzymes between 72 and 96 h were examined in detail. The activities of ICL and MAS first increased and then decreased (Fig. 4A and B), which indicated that sodium malonate could enhance the key enzyme activity of the glyoxylate cycle in a short period of time, allowing increased malate synthesis via this pathway. However, the activities of IDH and FUM first decreased and then increased (Fig. 4C and D), which further indicated that the competitive inhibition of sodium malonate was eliminated overtime.

Analysis of intracellular metabolites in the TCA cycle and glyoxylate cycle

To further analyze the biosynthetic pathway of malate in GXZ-6, the effects of citric acid and sodium malonate on the intracellular metabolites (citrate, succinate, fumarate, glyoxylate, and malate) were also investigated. As shown in Table 1, the PMA titer and biomass increased by 5.1% and 25.9%, respectively, when GXZ-6 was cultured for 48 h with the addition of citric acid at the beginning of fermentation. The concentration of intracellular citrate increased greatly from 2.93 ± 0.19 mg/g biomass to 45.87 ± 0.32 mg/g biomass. Interestingly, fumarate accumulation was observed, and the concentration of fumarate increased by 233.3%, but the glyoxylate concentration increased by only 9.2%. In addition, the malate concentration increased by 108.4%. These results indicated that malate was more likely to be derived from glyoxylate than from fumarate. Sodium malonate was added to the fermentation medium after 72 h of fermentation, and then, the GXZ-6 cells were cultured for 96 h. Succinate accumulation was observed, and the succinate concentration increased from 1.33 ± 0.18 mg/g biomass to 2.49 ± 0.84 mg/g biomass due to the inhibition of succinate dehydrogenase. Furthermore, the fumarate concentration did not change, but the glyoxylate concentration decreased by 22.3%. This decrease in glyoxylate levels might have been mainly due to malate synthesis, as the malate concentration increased from 2.63 ± 0.03 mg/g biomass to 4.6 ± 0.51 mg/g biomass. The above results also indicated that the glyoxylate cycle was more active than the TCA cycle.

Conclusion

The biosynthesis of malate for PMA production in *A. melanogenum* GXZ-6 involved the TCA cycle and glyoxylate cycle. The results of the addition of exogenous metabolic intermediates and inhibitors suggested that malate was synthesized significantly via the glyoxylate cycle rather than the TCA cycle. Furthermore, the effects of exogenous metabolic intermediates and inhibitors on malate biosynthesis were further studied at the metabolic, enzymatic, and transcriptional levels by analyzing the concentrations of key intracellular metabolites, key enzyme activities and transcription of the genes encoding key enzymes in the TCA cycle and glyoxylate cycle, providing novel insights into the biosynthesis of malate for PMA production in *A. pullulans*.

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