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Research Article

Toward understanding the key enzymes involved in β-poly (L-malic acid) biosynthesis by *Aureobasidium pullulans* ipe-1

β-poly (L-malic acid) (PMLA) is a biopolyester which has attracted industrial interest for its potential application in medicine and other industries. A high dissolved oxygen concentration (DO) was beneficial for PMLA production, while the mechanisms of DO in PMLA biosynthesis by *Aureobasidium pullulans* are still poorly understood. In this work, the amount of PMLA was first compared when *A. pullulans* ipe-1 were cultured under a high DO level (70% saturation) and a low DO level (10% saturation). Meanwhile, the key enzymes involved in different pathways of the precursor L-malic acid biosynthesis were studied. The results revealed that the activities of glucose-6-phosphate dehydrogenase (G6PDH) and phosphoenolpyruvate carboxylase (PEPC) were positively correlated with cell growth and PMLA production, while the activities of phosphofructokinases (PFK), pyruvic carboxylase (PC) and citrate synthetase (CS) did no show such correlations. It indicated that the Pentose Phosphate Pathway (PPP) may play a vital role in cell growth and PMLA biosynthesis. Moreover, the precursor L-malic acid for PMLA biosynthesis was mainly biosynthesized through phosphoenolpyruvic acid (PEP) via oxaloacetate catalyzed by PEPC. It was also found that low concentration of sodium fluoride (NaF) might impel carbon flux flow to the oxaloacetate through PEP, but inhibit the flux to the oxaloacetate via pyruvic acid.

Keywords: *Aureobasidium pullulans* / Biosynthesis mechanism / β-poly (L-malic acid) / Glucose-6-phosphate dehydrogenase / Phosphoenolpyruvate carboxylase

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1 Introduction

β-poly (L-malic acid) (PMLA) is a biopolyester which has attracted industrial interest for its potential application in medicine and other industries. In addition, PMLA can be easily hydrolyzed into malic acid, a dicarboxylic acid widely used in food and cosmetic industries [1, 2]. For its biosynthesis, most strains of *Aureobasidium pullulans* in genetically diverse phylogenetic clades could produce PMLA [3], and certain clades included strains that produced relatively high levels of PMLA up to 116.3-144.2 g/L [2, 4]. However, to date, PMLA has not been commercially produced by fermentation due to its high production cost and unclear biosynthesis mechanism.

For PMLA, its biosynthesis was supposed to be closely related to L-malic acid production. With the aid of NMR studies of D-[1-^{13}C] glucose and $Ca^{13}CO_3$, Lee et al. [5] found that L-malate used for PMLA production was synthesized either via carboxylation of pyruvate and reduction of oxaloacetate (OAA) in the presence of $CaCO_3$, or via the oxidative TCA cycle and most likely the glyoxylate shunt in the absence of $CaCO_3$. However, they did not detect the key enzymes involved in biosynthesis of L-malic acid. Moreover, it was reported that the suppression of mitochondrial activity could enhance malic acid production [6], and the mitochondrial activity could be affected by oxygen supply [7]. Meanwhile, Cao et al. [8] found that a high dissolved oxygen (DO) level was beneficial for PMLA production. Moreover, other products biosynthesized by *A. pullulans,*

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Abbreviations: PEP, phosphoenolpyruvic acid; **PEPC**, phosphoenolpyruvate carboxylase; **PMLA**, β-poly(L-malic acid); **PPP**, Pentose Phosphate Pathway

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such as pullulan [9–12] and biosurfactant [13], were all affected by DO level. However, they did not identify the specific enzymes participated in the biosynthesis. Usually, increased growth rate and aerobic metabolism alter the expression of many enzyme. In addition, the substrate could be transferred into the structural unit through central metabolic pathways generally consisted of Embden Meyerhof Pathway (EMP), Pentose Phosphate Pathway (PPP) and Entner–Doudoroff Pathway (EDP) for the cell's physiological activities and product synthesis [14]. However, whether all these three pathways participated in the PMLA biosynthesis is still unclear. Therefore, if the key enzymes which were positively associated with the PMLA production under different DO levels could be identified, it would improve the understanding of PMLA biosynthesis mechanism by *A. pullulans*.

In this study, we firstly aimed to find the key enzymes affecting the cell growth and PMLA production under a higher DO level (70% saturation) and a lower DO level (10% saturation). Then, some metabolic inhibitors and metabolic intermediates related to L-malic acid biosynthesis were used to regulate PMLA biosynthesis by manipulating the enzymatic activity. At last, the results in this study would be expected to clarify the mechanism of DO concentration in PMLA biosynthesis by *A. pullulans*.

2 Materials and methods

2.1 Microorganism

A. pullulans ipe-1 (CGMCC No. 3337) used in this study was stored in China General Microbiological Culture Collection Center, Beijing, China. The strain was maintained on potato-glucose agar slant at 4°C.

2.2 Culture medium

The seed culture medium contained per liter (w/v): 80 g glucose, 2 g $NaNO_3$, 0.1 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.5 g KCl and 1 g tryptone (Oxoid LP0042) in deionized water. For the production of PMLA in a 2.7 L bioreactor (BioFio® 110), the following medium was used per liter (w/v): 160 g glucose, 15 g tryptone, 2 g $NaNO_3$, 0.1 g KH_2PO_4 0.2 g $MgSO_4{\cdot}7H_2O$ and 0.5 g KCl in deionized water. The fermentation medium in flasks was the same as that in the 2.7 L bioreactor except for 30 g/L $CaCO_3$.

2.3 Culture method

The seed culture of strain ipe-1 was prepared by inoculating cells grown on potato-glucose agar into 500 mL Erlenmeyer flasks containing 100 mL of seed culture medium, followed by incubation at 25°C for 2 d on the rotary shaker. For flask culture, a 500 mL flask containing 50 mL medium was inoculated 5 mL of seed culture broth and cultured at 25°C for 5 d on the rotary shaker, then the final broth was diluted to 50 mL with deionized water for the relevant parameters measurement. For bioreactor culture, the seed culture broth (180 mL) was transferred to a 2.7 L bioreactor containing 1620 mL medium, which had the same components with the 1800 mL fermentation medium, and the total initial fermentation volume was 1800 mL. The temperature, aeration rate and pH in the bioreactor was kept at 25°C, 1–5 L/min and 6.0, respectively, and the pH was kept constant automatically by adding 2 M NaOH or 1 M H_2SO_4. When dissolved oxygen concentration (DO) dropped to a set value, the stirring speed was automatically controlled based on the value of DO concentration, which was detected by a DO electrode. All fermentation experiments were performed at least twice, to ensure the observed trends were correct and reproducible.

2.4 Analytical methods

The culture broth (8 mL) was centrifuged at 10 000 × *g* for 8 min and the resulting supernatants were used for the measurement of PMLA and glucose. For the measurement of biomass, the cells were washed three times with 8 mL distilled water, then dried to constant weight at 90°C. To measure the amount of PMLA, 1 mL supernatants were incubated with 1 mL 2 M H_2SO_4 for 12 h at 90°C. After neutralization of the solution, L-malic acid concentration was measured by a HPLC apparatus (Shimadzu LC20AT) equipped with a Aminex HPX-87H column. Glucose concentrations of the supernatants were measured using a biosensor with glucose oxide electrodes (Institute of Biology, Shandong Academy of Science SBA-40C). The kits used for measuring enzyme activity of glucose-6-phosphate dehydrogenase (G6PDH), 2-keto-3-deoxy-6-phosphogluconate aldolase (KDPG), phosphoenolpyruvate carboxylase (PEPC) and pyruvic carboxylase (PC) were purchased from Suzhou Comin Biotechnology Co. Ltd (Suzhou, China). And the kits used for measuring enzyme activity of citrate synthetase (CS) and phosphofructokinases (PFK) were purchased from Nanjing Jiancheng Biotechnology Institute (Nanjing, China). The kit used for measuring protein concentration was purchased from Beyotime (Shanghai, China). The measurement processes were followed as the product specification. The enzyme activity (U) was expressed in in nmol per min per mg protein. The endogenous ATP was measured using an ATP Assay Kit (Abnova KA1661).

The batch fermentation data were used to determine four important parameters describing cell growth and PMLA formation kinetics. The cell yield ($Y_{x/s}$) was determined from the slope of the plot of cell density versus consumed glucose concentration. The PMLA yield ($Y_{p/s}$) was determined from the slope of the plot of PMLA formation versus consumed glucose concentration. The specific PMLA production per unit cell mass ($Y_{p/x}$) was estimated from the slope of the plot of PMLA formation versus increased cell mass. The PMLA productivity was calculated from the slope of the plot of PMLA concentration versus the production time.

3 Results

3.1 Effect of DO on enzymes activity involved in PMLA biosynthesis

Effect of DO on enzymes activity involved in PMLA biosynthesis under a high DO level (70% saturation) and a low DO level (10% saturation) was first examined. The key enzymes

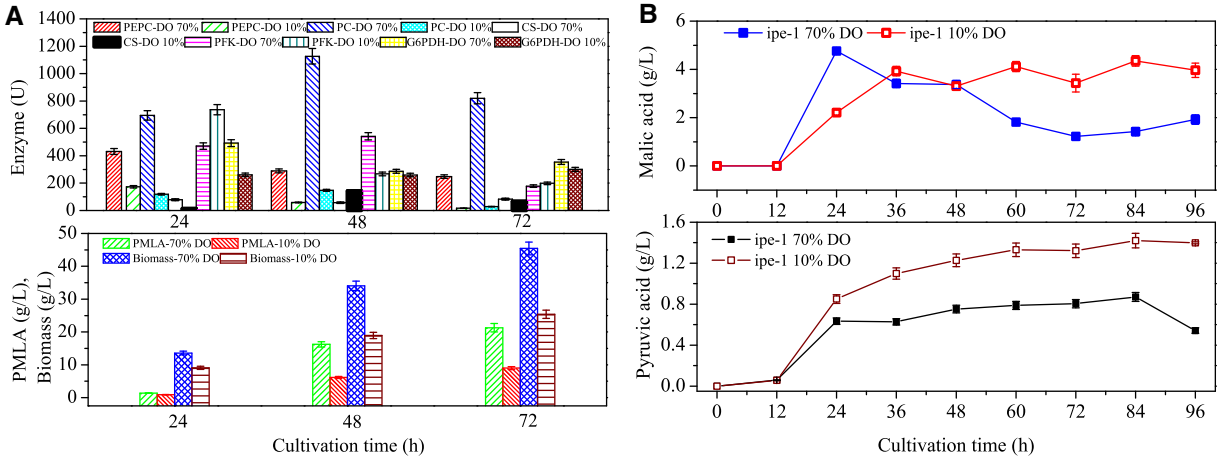

Figure 1. Effect of DO levels on the key enzymatic activity, PMLA, and biomass (A), and the main metabolic intermediates (i.e. malic acid and pyruvic acid) (B). Data are average values and standard deviations of triplicate experiments.

associated with L-malic acid biosynthesis was examined after culturing for 18 h, since DO was maintained at constant level after that time and L-malic acid was regarded as the direct precursor for PMLA biosynthesis. As shown in Fig. 1A, the activities of PFK (a rate-limiting enzyme in the EMP) and G6PDH (the first step and crucial check point of the PPP) existed in *A. pullulans* ipe-1, whereas KDPG activity (a specific enzyme existed in the ED) was not detected. Therefore, during PMLA biosynthesis by *A. pullulans* ipe-1, it indicated that the PPP and EMP might play a vital role, while the the ED did not work. Moreover, the activity of G6PDH was positively proportional to PMLA production, while the activity of PFK did no show such correlation. Thus, the PPP may be more important for PMLA biosynthesis than that of EMP. Moreover, PC, catalyzing pyruvic acid for biosynthesis of oxaloacetic acid, and PEPC, catalyzing phosphoenolpyruvic acid (PEP) for biosynthesis of oxaloacetic acid, also showed positively correlation with PMLA production, while CS (a key enzyme of TCA) did not. Moreover, the key enzymes involved in PMLA biosynthesis was shown in Fig. 2. Therefore, it is required to investigate the effect of the metabolic intermediates or inhibitors associated with the key enzymes activity on PMLA biosynthesis for understanding the biosynthesis mechanism of PMLA production. Meanwhile, excess L-malic aid, which is the direct precursor for PMLA biosynthesis (Fig. 1B), could be detected in the broth, indicating that the precursor biosynthesis was not a limiting factor for PMLA biosynthesis. In fact, the PMLA polymerization and depolymerization happened simultaneously [15, 16], and PMLA hydrolase was responsible for this depolymerization [17]. Gasslmaier and Holler [18] reported that the catalytic mechanism of the PMLA hydrolase dominated the substrate-binding specificity and the depolymerization proceeded from the hydroxy-terminus towards the carboxyterminus, thereby degrading the polymer to L-malate. Therefore, the amount of malic acid in the broth would be varied during PMLA biosynthesis. Another intermediate metabolite pyruvic acid was also excessive in the broth, while whether PMLA biosynthesis could be regulated by pyruvic acid was still unknown which needed to be further investigated. In addition, PMLA production, cell growth and glucose consumption were positively correlated with DO concentration. At the final batch culture (Table 1), better fermentation efficiency (i.e. 23.6 g/L PMLA and 54.9 g/L biomass) was obtained at 70% DO. Thus, the experiments in the next sections would be carried out at 70% DO except elsewhere stated.

3.2 Effect of pyruvic acid on PMLA biosynthesis

As showed in Fig. 2, pyruvic acid was a key metabolic node in the biosynthesis of PMLA, while there is excess pyruvic acid detected in the broth during PMLA biosynthesis. Therefore, effect of exogenous pyruvic acid on PMLA biosynthesis was firstly examined in flasks (Fig. 3A). With the increasing addition of exoge-

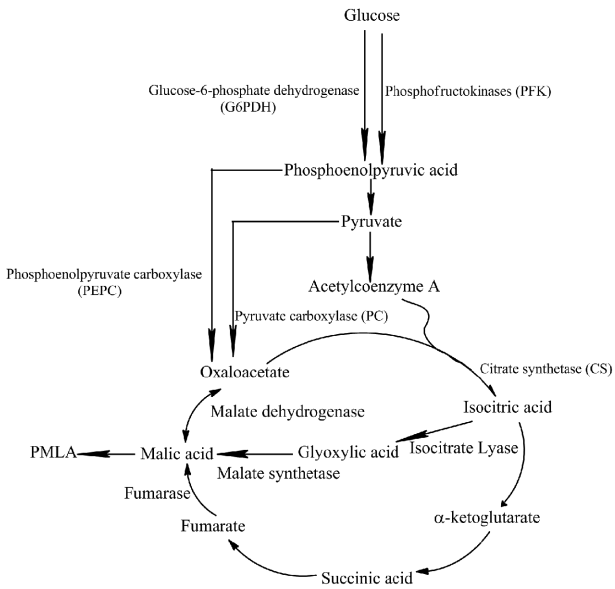

Figure 2. Schematic mechanism of the key enzymes involved in PMLA biosynthesis.

Table 1. Effect of operation strategies on the key enzymes in pathway of PMLA biosynthesis

Operation strategy [a)]	PEPC (U)	PC (U)	CS (U)	Biomass (g/L)	PMLA (g/L)	$Y_{p/x}$	$Y_{p/s}$	$Y_{x/s}$
70% DO	247.9 ± 26.5	820.0 ± 74.6	83.0 ± 7.5	54.9 ± 2.4	23.6 ± 1.1	0.43	0.14	0.33
10% DO	16.6 ± 1.5	29.0 ± 2.3	66.7 ± 5.9	34.8 ± 1.8	10.8 ± 0.4	0.31	0.10	0.21
Pyruvic acid	233.0 ± 27.2	272.4 ± 24.3	48.6 ± 4.6	50.3 ± 2.1	27.8 ± 1.2	0.55	0.17	0.30
NaF	366.8 ± 33.4	673.9 ± 58.3	68.7 ± 7.1	50.0 ± 2.3	26.7 ± 1.4	0.53	0.16	0.31
25 mmol/L TFA	477.7 ± 45.8	245.3 ± 19.2	89.4 ± 8.7	56.5 ± 2.6	24.1 ± 1.0	0.43	0.15	0.35
100 mmol/L TFA	181.2 ± 16.4	274.4 ± 22.5	13.4 ± 1.1	33.4 ± 1.6	14.8 ± 0.9	0.44	0.12	0.26
ATP	567.5 ± 48.9	362.3 ± 31.8	23.8 ± 2.5	45.7 ± 1.9	26.6 ± 0.9	0.58	0.17	0.29
100 mmol TFA+ATP	230.0 ± 21.5	373.9 ± 36.2	11.5 ± 1.4	31.4 ± 1.4	17.4 ± 0.8	0.55	0.13	0.24

[a)] The activity of enzymes was measured after culturing for 72 h. $Y_{p/s}$, $Y_{p/x}$ and $Y_{x/s}$ in the table are mean values.

nous pyruvic acid, the produced L-malic acid and the remaining pyruvic acid in the broth increased, while cell growth showed a little decrease. For PMLA production, the PMLA concentration increased with the increasing addition of pyruvic acid below 5 g/L, while further increasing pyruvic acid above that concentration would inhibit PMLA biosynthesis. Second, in order to verify the effect of pyruvic acid on PMLA biosynthesis, 5 g/L pyruvic acid was added in the bioreactor at 36 h (Fig. 3B) since the precursor L-malic acid began to decrease from that point (Fig. 1B). During the fermentation process, the added exogenous pyruvic acid could be assimilated by the strain ipe-1, while the rate of glucose consumption decreased. The resulting PMLA concentration reached 27.8 g/L, which was increased by 17.8% (but biomass was decreased by 8.4%) compared with the batch culture without the addition of pyruvic acid (Table 1). Meanwhile, the malic acid concentration was continuously decreased until 96 h from cultivation for 24 h, which was different from that in Fig. 1B. Third, to verify whether the increase of PMLA production was caused by the metabolism promotion of exogenous pyruvic acid, extra glucose (final concentration 40 g/L) was added in the broth at 72 h to guarantee enough carbon source. As shown in Fig. 3C, in the end of culture, the concentration of PMLA was 27.2 g/L, which was about the same as that in Fig. 3B. Meanwhile, the malic acid concentration was about constant between 60 h and 96 h, which was different from that in Fig. 1B. Therefore, the enhancement of PMLA production caused by the addition of exogenous pyruvic acid was not due to the metabolic enhancer of pyruvic acid, which only acted as an extra carbon source in this case. Moreover, the increased concentration of malic acid in Fig. 1B between 60 h and 96 h may be caused by the depolymerization of PMLA, since the PMLA molecular weight decreased in the late exponential growth phase in our precious work when the glucose concentration was low [15]. When exogenous pyruvic acid was added in the broth, the rate of glucose consumption declined which decreased the malic acid production from glucose, meanwhile some malic acid was used for PMLA biosynthesis by the strain ipe-1. Thus, the malic acid concentration decreased continuously (Fig. 3A). However, when glucose was fed in the broth, more malic acid was synthetized from glucose, where the rate of malic acid used for PMLA biosynthesis may be equal to the malic acid production from glucose, and that was possibly why the malic acid concentration was about constant between 60 h and 96 h (Fig. 3B).

3.3 Effect of sodium fluoride on PMLA biosynthesis

Since exogenous pyruvic acid could not be used as a metabolic enhancer for PMLA biosynthesis, whether PMLA production could be regulated by decreasing biosynthesis of endogenous pyruvic acid would be examined. It was reported that sodium fluoride (NaF) could inhibit pyruvate kinase to decrease cellular pyruvate and accumulation of PEP [19]. Therefore, NaF was used to decrease endogenous pyruvic acid. As shown in Fig. 4A, with the increasing addition of NaF, the produced pyruvic acid decreased, while cell growth (26.8 g/L) was not significantly affected. Meanwhile, the produced PMLA and L-malic acid increased with the increasing addition of NaF below 0.18 g/L, while further increasing NaF above that concentration would inhibit the biosynthesis of PMLA and L-malic acid. Considering that the biomass in bioreactor was about twice of that in flask, the highest NaF concentration (0.3 g/L) was added in the bioreactor. As a result (Fig. 4B), 26.7 g/L PMLA was obtained which was increased by 13.1% compared with that in Fig. 1. Moreover, the activity of PEPC was increased by 48.0%, while the activity of PC and CS were respectively decreased by 17.8 and 17.2% (Table 1). Therefore, PMLA biosynthesis could be promoted by decreasing endogenous pyruvic acid though the PC activity was high, which also indicated that PMLA biosynthesis was mainly catalyzed by PEPC rather than catalyzed by PC.

3.4 Effect of sodium trifluoroacetate on PMLA biosynthesis

Although citrate synthetase (CS) activity was not closely associated with PMLA biosynthesis (Fig. 1), it was a key enzyme in citric acid cycle (TCA) (Fig. 2). It may be a good way to examine the role of TCA and glyoxylate shunt on PMLA biosynthesis by regulating the CS activity. It was reported that trifluoroacetic acid (TFA) could inhibit the CS activity [20, 21]. Thus, the effect of different concentration of TFA on PMLA biosynthesis was examined. In flasks, the produced PMLA started to decrease when the addition concentration of TFA was above 15 mmol/L (Fig. 5A). In order to further verify the effect of TFA on PMLA biosynthesis, higher concentration of TFA was added in the broth at initial stage of fermentation. With addition of 25 mmol/L TFA, the concentration of PMLA and cell growth were about the same

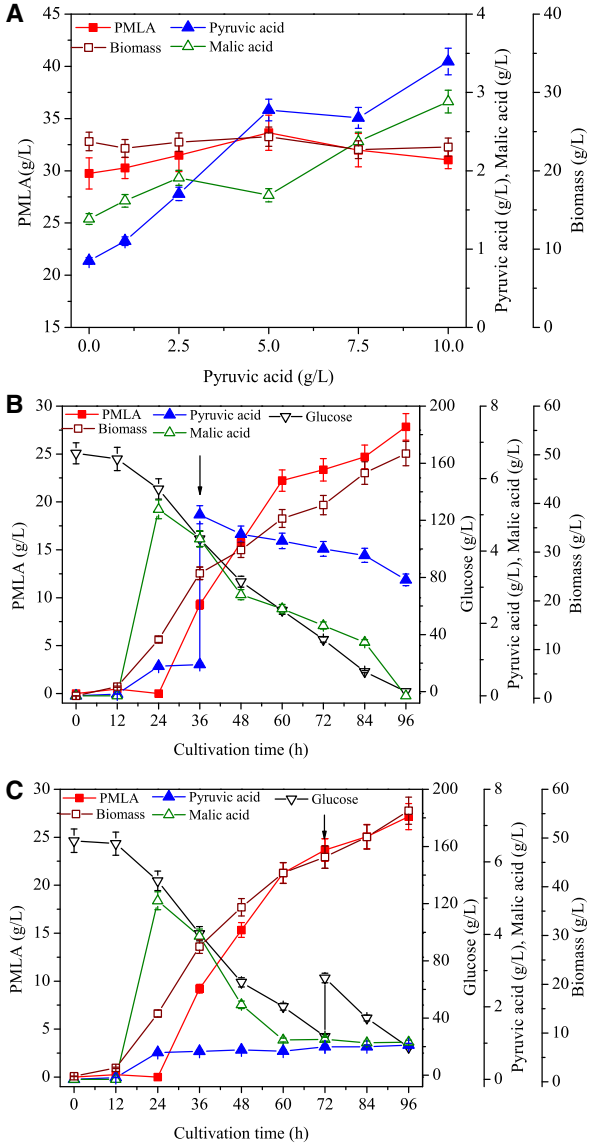

Figure 3. Effect of pyruvic acid concentration on fermentation (A), kinetics of PMLA production with an addition of pyruvic acid (finial concentration 5 g/L) at 36 h (B) and kinetics of PMLA production with an extra addition of glucose (final concentration 40 g/L) at 72 h (C). The results in Fig. 3 A was obtained in the experiment with 500 mL flasks, and the others were from the experiments with a 2.7 L bioreactor. Data are average values and standard deviations of two experiments.

as those in Fig. 1. Further enhancing the addition concentration of TFA to 100 mmol/L, the concentration of PMLA and cell growth were respectively decreased by 37.3 and 39.2% compared with those in Fig. 1. Meanwhile, the activity of CS, PEPC and PC were respectively decreased by 83.5, 26.9 and 66.5%. Moreover, L-malic acid concentration was higher in the broth with the addition of 100 mmol/L TFA, indicating that L-malic acid could not be effectively converted into PMLA under this condition. In view of the TCA, ATP was one important product from TCA whose yield could be regulated by regulating the

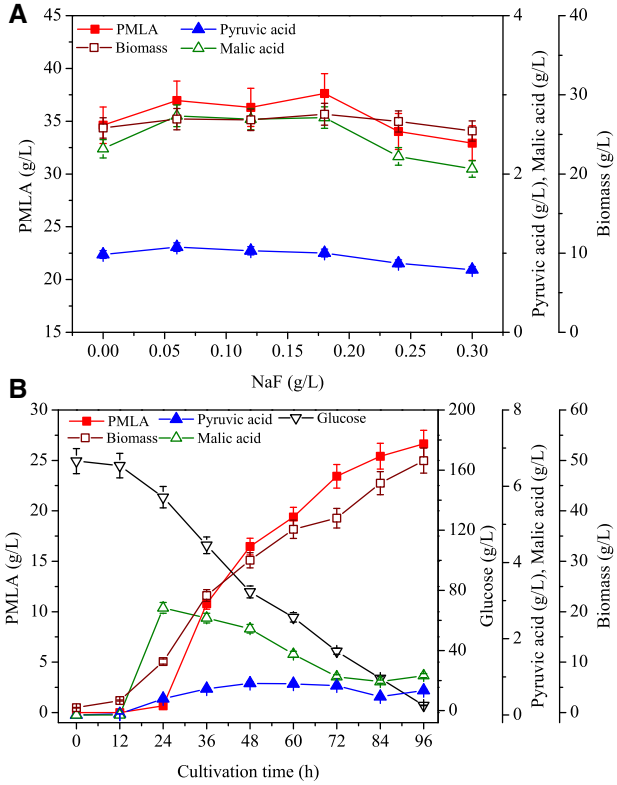

Figure 4. Effect of NaF concentration on fermentation in a 500 mL flask (A), and kinetics of PMLA production with 0.3 g/L NaF concentration in a 2.7 L bioreactor (B). NaF was added at the initial fermentation phase. Data are average values and standard deviations of two experiments.

activity of CS. Therefore, the effect of ATP on PMLA biosynthesis would be examined.

3.5 Effect of ATP on PMLA biosynthesis

To verify the effect of ATP on PMLA biosynthesis, 0.5 g/L exogenous ATP was added in the broth at 36 h. As showed in Fig. 6A, the PMLA concentration was increased by 12.7%, while the cell growth was decreased by 16.8% compared with those in Fig. 1. Meanwhile, the activity of CS and PC were respectively decreased by 71.3 and 55.8%, while PEPC activity was increased by 1.3 times. Compared to the case without ATP adding, the the endogenous ATP was respectively increased by 10.7 and 14.2% at 48 and 72 h. These results confirmed that PMLA biosynthesis was closely correlated with PEPC activity, and cell growth was partly correlated with CS activity. In addition, because of the decreased activity of CS and PC, pyruvic acid was higher in the broth before 96 h compared with that in Fig. 1. It indicated that the addition of exogenous ATP could also inhibit TCA and decrease cell growth. However, PEPC activity was enhanced which promoted the PMLA production. To further verify the effect of ATP on PMLA biosynthesis, co-feeding ATP and TFA was carried out. Compared with those in Fig. 5B, PMLA concentration was increased by 17.6%, and cell growth was decreased by 6.0% in

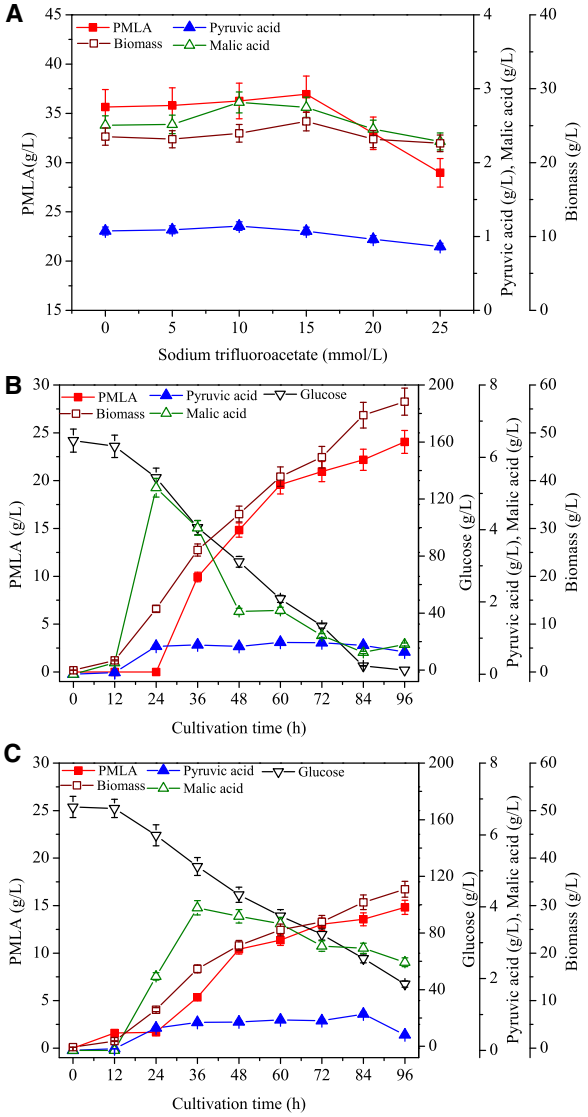

Figure 5. Effect of sodium trifluoroacetate (TFA) on fermentation (A), kinetics of PMLA production with 25 mmol/L TFA (B) and 100 mmol/L TFA (C). The results in Fig. 3 A was obtained in the experiment with 500 mL flasks, and the others were from the experiments with a 2.7 L bioreactor. The TFA was added at the initial fermentation phase. Data are average values and standard deviations of two experiments.

Fig. 6B. Meanwhile, CS was also decreased 14.2%, while PEPC was increased by 27.6%. The results confirmed that PMLA biosynthesis was positively correlated with the PEPC activity, and ATP participated in PMLA biosynthesis.

4 Discussion

PMLA can be largely produced by microbial fermentation, while the mechanisms of PMLA biosynthesis are still unclear. However, L-malate was widely verified and accepted as the precursor for PMLA biosynthesis [5, 22, 23]. Meanwhile, a high DO level was beneficial for PMLA production [8, 24]. Therefore, the key enzymes activities involved in the precursor production for PMLA biosynthesis was studied under a high DO level (70% saturation) and a low DO level (10% saturation). Through investigating the effects of DO on PMLA biosynthesis in this study, we found that PMLA production was positively associated with the activity of PEPC, while it did not show such association with PC and CS. Moreover, PMLA production could not be increased by increasing the concentration of the direct substance pyruvic acid of PC (though PC activity was high). However, PMLA concentration could be increased by the addition of NaF, which could not only decrease the endogenous pyruvate but also accumulate phosphoenolpyruvic acid (PEP) [19]. The accumulated PEP could be catalyzed by PEPC to synthesize oxaloacetic acid, and further to synthesize L-malic acid. In addition, under fermentative conditions, PEPC also has a catabolic function: it determines a portion of the PEP to succinic acid [25], which can interpret why a high concentration of TFA (even up to 25 mM) would not inhibit PMLA biosynthesis in bioreactor. Whereas, when the PEPC converted PEP to oxaloacetate, no ATP is produced, leading to losing of the high-energy phosphate bond of PEP. Therefore, there are some problems for the strain ipe-1 to use PEPC for production of PMLA.

By studying the effect of metabolic inhibitors and organic acids on the production of PMLA, Liu and Steinbüchel [20]

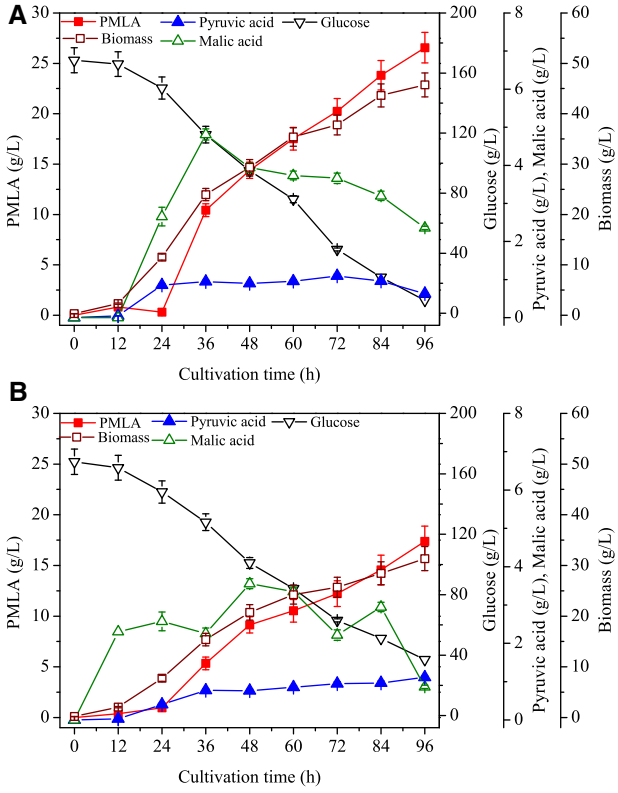

Figure 6. Kinetics of PMLA production with 0.5 g/L ATP (A) and co-feeding of TFA and ATP (B) in a 2.7 L bioreactor. The ATP was added at 36 h, and the TFA was added at the initial fermentation phase. Data are average values and standard deviations of two experiments.

speculated that PMLA synthesis was probably related to the TCA and also indicated that isocitrate lyase and malate synthase participated in PMLA biosynthesis. Moreover, we found that PMLA production could be enhanced by adding exogenous ATP. Meanwhile, a high concentration TFA (25 mmol/L) could not inhibit PMLA production, and co-feeding ATP and more higher concentration of TFA (100 mmol/L) could mitigate the inhibition of TFA, which indicated that PMLA biosynthesis needed the participation of ATP. These results explained why high DO level was beneficial for PMLA biosynthesis since large ATP could be produced at high DO level. Li et al. [26] also verified that large ATP could be produced by the yeast *Torulopsis glabrata* in pyruvic acid production process at high DO levels. Therefore, when oxaloacetate was biosynthesized from PEP catalyzed by PEPC, large amount of ATP could be produced by the strain ipe-1 at high DO level for replenishing the high-energy phosphate bond of PEP, and the elevated ATP levels could inhibit the glycolytic cycle because the key enzymes of glycolysis, phosphofrucotokinase (PFK) could be inhibited [26]. However, the strain ipe-1 possessed high activity of glucose-6-phosphate dehydrogenase (G6PDH), and thus PPP played a vital role in PMLA biosynthesis when EMP was inhibited. The work by Sheng et al. [14] also confirmed that the central metabolic pathways of *A. pullulans* included EMP and PPP. Similar as NaF, another inhibitor iodoacetic acid also could impel carbon flux flow toward the PPP [14]. The PPP would be important for cell growth, because the ribose derived from PPP is precursor of nucleic acid and the cell growth is actually correlated with the G6PDH activity. Fig. 1 also shows that high cell growth in high DO level could be achieved. In fact, it was found that PMLA production was associated with cell growth in the early exponential growth phase and could be enhanced by maintaining cell growth in the exponential growth phase [27]. Furthermore, we found that the activity of G6PDH was positively correlated with PMLA production, while the activity of PFK did no show such correlation. Therefore, during the biosynthesis process of PMLA, glucose consumption rate was rapid which was not inhibited by the elevated ATP levels, and also little affected by the TFA (below 25 mmol/L).

Therefore, the results in this study verified that the precursor L-malic acid was mainly produced through PEP catalyzed by PEPC, and large amount of ATP should be produced at high DO level for replenishing the high-energy phosphate bond of PEP. It was confirmed that PEPC, which was produced during growth on glycolytic substrates and capable of carboxylating PEP to produce oxaloacetate, functioned aerobically to replenish oxaloacetate consumed in biosynthetic reactions [28]. Several carbohydrates were found to be taken up in *Salmonella typhimurium* and *Escherichia coli* by the PEP carbohydrate phosphotransferase system (PTS) [29]. During their transport across the membrane, the substrates were phosphorylated. The phosphoryl groups derived from PEP, and were transferred to different carbohydrates by a number of proteins, most of which were substrate specific. Moreover, the highest level of PMLA concentration (144.2 g/L) and PMLA productivity (1.15 g/L·h) produced with A. pullulans have been reported by Zou et al. [2] and Cao et al. [27], respectively. In the present study, both PMLA concentration (26.56 g/L) and productively (0.28 g/L·h) were quite low. However, our objective here is to understand the key enzymes involved in PMLA biosynthesis by A. pullulans, which is not only used for process development and optimization of PMLA production, but also supplies some gene manipulation sites for further enhancing PMLA production.

5 Concluding remarks

This paper revealed the proper metabolic pathway of PMLA produced from *A. pullulans* ipe-1. During the PMLA biosynthesis, PPP and EMP played a vital role, while the ED did not work. The activity of G6PDH and PEPC were positively associated with PMLA production, while the activity of PFK, PC and CS did no show such correlation. Thus, the PPP might be more important for PMLA biosynthesis than that of EMP. Furthermore, the precursor L-malic acid for PMLA biosynthesis was biosynthesized through PEP via oxaloacetate catalyzed by PEPC, and large amount of ATP should be produced at high DO level for replenishing the high-energy phosphate bond of PEP. Moreover, the findings also revealed that a low concentration of NaF might impel carbon flux flow to the oxaloacetate through PEP, but depress the flux to the oxaloacetate through pyruvic acid. Therefore, the findings in this study would not only provide a good reference for process development and optimization of PMLA production, but also supply some gene manipulation sites for enhancing PMLA production.

Practical application

β-poly(L-malic acid) (PMLA) is a biopolyester which has attracted growing attention due to its potential applications in medicine and other industries. It has versatile pendant carboxy group which allows introducing biologically active molecules and/or a targeting moiety via appropriate chemical modifications. In this work, it was found that the Pentose Phosphate Pathway (PPP) play a vital role in cell growth and PMLA biosynthesis. Moreover, the precursor L-malic acid for PMLA biosynthesis was mainly biosynthesized through phosphoenolpyruvic acid (PEP) via oxaloacetate catalyzed by phosphoenolpyruvate carboxylase (PEPC). It was also found that low concentration of sodium fluoride (NaF) might impel carbon flux flow to the oxaloacetate through PEP, but inhibit the flux to the oxaloacetate via pyruvic acid. Therefore, the findings in this study would not only provide a good reference for process development and optimization of PMLA production, but also supply some gene manipulation sites for enhancing PMLA production.

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