1 **High molecular weight β -poly(L-malic acid) produced by *A.***
2 ***pullulans* with Ca^{2+} added repeated batch culture**

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1 **Abstract**

2 β -Poly(malic acid) (PMLA) has attracted increasing attentions because of its
3 potential application in medicine and other industries. In this study, the variation
4 of PMLA molecular weight (M_w) in the batch culture and the strategies to enhance
5 PMLA M_w were studied. Adding exogenous Ca^{2+} (0.1 g/L $CaCl_2$) to the medium
6 caused a significant increase in both PMLA concentration and M_w (11.38% and
7 26.3%, respectively) when Na_2CO_3 was used as the neutralizer. The M_w of PMLA
8 during the process of batch culture, which associated with the specific PMLA
9 production per unit cell mass ($Y_{p/x}$) before glucose was depleted, increased from
10 12.522KDa to its maximum 18.693KDa and then kept decreasing until the end of
11 the culture. Compared with the results in batch culture, M_w increased by 84.4%
12 (up to 19.51kDa) with a productivity of $1.1 \text{ g}\cdot\text{h}^{-1}\cdot\text{L}^{-1}$ when the cells were
13 maintained in exponential growth phase during Ca^{2+} added repeated batch culture.
14 The present work provides an efficient approach to obtain superior quality PMLA
15 product with high M_w .

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17 **Keywords** β -poly(L-malic acid), *Aureobasidium pullulans*, molecular weight

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1 **1. Introduction**

2 β -Poly(malic acid) (PMLA) is a biodegradable, biocompatible and water
3 soluble natural biopolyester with versatile pendant carboxy group, which allows
4 the conjunction of biologically active molecules and/or a targeting moiety via
5 appropriate chemical modifications [1, 2]. PMLA has attracted increasing
6 attentions because of its potential application in medicine and other industries [3,
7 4]. It could be biosynthesized by the *Physarum polycephalum* and *A. pullulans*.
8 For *P. polycephalum*, the low concentration of PMLA (2.7 g/L) in broth [5] has
9 restricted its further application for large-scale production of PMLA. While most
10 strains of *A. pullulans* in genetically diverse phylogenetic clades could produce a
11 high concentration of PMLA [6, 7], from 9.8 g/L [8] to 87.6 g/L [9] in free-cell
12 fermentation with a stirred bioreactor. Therefore, *A. pullulans* was a promising
13 microorganism for the industrial production of PMLA in the near future.

14 Besides the final concentration, PMLA molecular weight (M_w) is also an
15 important factor affecting the PMLA applications. For example, PMLA with M_w
16 of 5KDa could be used as a protease inhibitor [10], while PMLA with M_w of
17 50KDa was used for synthesizing a new prototype of polymer-derived drug
18 delivery system [11]. A minimal chain length of approximately 10 malate residues
19 was required to inhibit homologous DNA polymerase α [12], and an intravenous
20 PMLA nanobioconjugate (at least contain 8 malate residues) was used for
21 inhibition of brain tumor growth [13]. Therefore, it is necessary to regulate PLMA
22 M_w during PMLA biosynthesis. In this regard, different molecular weights of final
23 PLMA products from 4.6 to 11 kDa by *A. pullulans* and *Aureobasidium sp.* strains
24 [6, 8, 14, 15] were obtained under different fermentation conditions, implying that
25 PLMA M_w can be controlled by optimizing the process parameters. However,
26 there has no report regarding the variations of PMLA M_w during PMLA

1 biosynthesis.

2 Although the available method for increase of PMLA M_w cannot be found in
3 the present literature yet, Nagata et al. [14] reported that the addition of CaCO_3 to
4 culture media could increase the PMLA concentration, and the M_w of obtained
5 PLMA was relatively high among the known results. Inspired by this, we
6 hypothesize that CaCO_3 addition during the fermentation can also increase the
7 PLMA M_w . However, the addition of CaCO_3 would produce various effects on the
8 PLMA fermentation. For instance, when the experiment was carried out in flasks,
9 the addition of CaCO_3 would change both ionic strength and pH during the
10 fermentation. When producing PMLA in bioreactor, the addition of CaCO_3 as
11 neutralizer could result in a highly viscous suspension because the cells interacted
12 with the precipitates, and this would have a detrimental effect on the rate of
13 oxygen transfer. Therefore, it is difficult to clarify the mechanisms how CaCO_3
14 addition did affect the PMLA production and to find suitable fermentation
15 conditions to regulate the M_w of PMLA products. To the best of our knowledge,
16 the variation of PMLA M_w during the batch culture and the strategies to enhance
17 PMLA M_w were not investigated yet.

18 In this work, different neutralizers were added during PMLA fermentations
19 in order to seek the real reason influencing PMLA production (including PMLA
20 concentration and M_w) with the addition of CaCO_3 , and then the relationship
21 among PMLA production, PMLA molecular weight and cell growth was
22 illustrated. The objective of this study is not only to clarify the effect of CaCO_3
23 addition on PMLA production, but also to establish an approach to produce high
24 M_w of PMLA by repeated batch culture.

25 **2. Materials and methods**

26 **2.1 Microorganism**

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

4 **2.2 Culture medium**

5 The compositions (w/v) of the seed culture medium were as follows: 8%
6 glucose, 0.2% NaNO₃, 0.01% KH₂PO₄, 0.02% MgSO₄·7H₂O, 0.05% KCl and
7 0.1% tryptone (LP0042, Oxoid LTD., Basingstoke Hampshire, England) in
8 deionized water. The compositions of the fermentation medium in flasks were the
9 same as the seed culture except for the CaCO₃ concentration. For the production
10 of PMLA in the 7.5L bioreactor (BioFio[®]110, New Brunswick Scientific, USA),
11 the following medium was used (w/v): 19% glucose, 1.5% tryptone, 0.6% NaNO₃,
12 0.5% yeast extract (LP0021, Oxoid LTD., Basingstoke Hampshire, England),
13 0.05% KH₂PO₄, 0.02% MgSO₄·7H₂O, 0.05% KCl and 5 ppm ZnSO₄·7H₂O in
14 deionized water.

15 **2.3 Culture method**

16 In all fermentation experiments, the seed culture of strain ipe-1 was prepared
17 by inoculating cells grown on potato-glucose agar into 500 mL Erlenmeyer flasks
18 containing 100 mL of seed culture medium, followed by incubation at 25 °C for
19 2d on the rotary shaker (HYG-A, Taicang Experimental Equipment Factory, China)
20 at 180 rpm. For flask culture, a 500 mL flask containing 50 mL medium was
21 inoculated with 5 mL of seed culture broth and cultured at 25 °C for 5 d on the
22 rotary shaker (180 rpm). The seed prepared for bioreactor was cultured in six
23 Erlenmeyer flasks each time, respectively. After culturing and mixing the seed
24 culture broth, 400 mL of the mixture was transferred to the 7.5 L bioreactor
25 containing 4 L of the fermentation culture medium. The temperature, aeration rate
26 and pH in the bioreactor was kept at 25 °C, 0.25-2.5 vvm and 6.0, respectively, and

1 the pH was kept constant automatically by adding alkali or 1 M H₂SO₄. DO was
2 controlled by agitation and detected by a DO electrode. When DO dropped to the
3 set value of 70%, the agitation rate was automatically increased based on the
4 value of DO concentration. For repeated-batch fermentation, when initial batch
5 fermentation arrived at the end of exponential growth, 3 L of the broth was
6 removed, and 3 L of the fresh medium was feed into the bioreactor. The
7 fermentation was restarted under the same conditions as the batch fermentation.
8 All fermentation experiments were performed at least twice, to ensure the
9 observed trends were correct and reproducible. Not all the data was presented in
10 the figures.

11 **2.4 Analytical Methods**

12 The culture broth (8 mL) was centrifuged in a high speed centrifuge (4-16 K,
13 Sigma, Germany) at 10000 g for 8 min and the resulting supernatants were used
14 for the measurements of PMLA, glucose and absorbance. For the measurement of
15 biomass, the cells were washed three times with 8 mL distilled water, dried to
16 constant weight at 90 °C. However, excess CaCO₃ was removed by the addition of
17 1 M HCl before the measurement of biomass in flask conditions. To measure the
18 amount of PMLA, 1 mL supernatants were incubated with 1 mL 2 M H₂SO₄ for
19 12 h at 90 °C. After neutralization of the solution, L-malic acid concentration was
20 measured by a HPLC apparatus (LC20AT, Shimadzu, Japan) equipped with a
21 SPR-H column (Shimadzu, Japan). The separation was performed at UV 210 nm,
22 a temperature of 40 °C, a flow rate of 0.6 mL/min and 4 mM perchloric acid was
23 used as eluant. Glucose concentrations of the supernatants were measured by a
24 biosensor with glucose oxide electrodes (SBA-40C, Biology Institute of Shandong
25 Academy of Sciences, China). To recover PMLA from broth, the procedures were
26 carried out as described in Nagata et al. [14]. The supernatant (5 ml, pH6.0) was

1 warmed to about 50 °C and MeOH (1.8 ml) was added dropwise with stirring to
2 the warmed supernatant. A resulting string precipitate of polysaccharide was
3 filtrated with 0.2 µm filter. MeOH (10 ml) was further added dropwise with
4 stirring to the mixture. The mixture was left 12 h with mild stirring at 4 °C. A
5 resulting precipitate was collected by filtration, suspended in deionized water (3
6 ml) and fully stirred. Small amounts of insoluble impurities were removed by
7 filtration. MeOH (6 ml) was added dropwise under stirring to the filtrate, and then
8 the mixture was left for 12 h at 4 °C. A resulting precipitate was collected by
9 filtration and washed successively with 60% MeOH. The pulverized and air-dried
10 precipitate was used in subsequent experiments. The PMLA M_w was evaluated by
11 gel-permeation chromatography using Waters apparatus with TSKgel
12 G3000PW_{XL} (Tosoh, Tokyo), which was calibrated with polyethylene glycol
13 standards (Tosoh, Tokyo and Polysciences, Inc., Warrington) under the following
14 conditions: flow rate, 1.0 mL/min; eluate, 0.2 M sodium phosphate buffer, pH6.8;
15 detector RI. Melanin was measured as described in Gadd et al. [16]. To determine
16 cellular melanin, washed cell pellets, obtained by centrifuging 5 ml culture
17 samples at 4000 g for 5 min, were autoclaved with 3 ml of 1 M NaOH (20 min,
18 120 °C). After cooling and centrifugation, 3 ml of borate buffer (pH 8.0) was
19 added to the supernatant and the absorbance at 540 nm was measured.
20 Extracellular melanin was determined by acidifying the cell-free culture
21 supernatants to pH2.0 with 1 M HCl and centrifuging at 10000 g for 10 min. Then
22 extracellular melanin was extracted with 1 M NaOH and the absorbance at 540
23 nm was measured as described previously. Extracellular polysaccharide was
24 isolated as described in Nagata et al. [21]. The reducing capacities (RC) of culture
25 filtrates were determined by ferri-cyanide reduction method and calculated with
26 the formula: $RC = [(A_{420} \text{ control} - A_{420} \text{ sample}) / A_{420} \text{ control}] \times 100\%$ [17].

1 Four important parameters were calculated as follow: the cell yield ($Y_{x/s}$) was
2 determined from the slope of the plot of cell density versus consumed glucose
3 concentration; the PMLA yield ($Y_{p/s}$) was calculated from the slope of the plot of
4 PMLA production versus consumed glucose concentration; the specific PMLA
5 production per unit cell mass ($Y_{p/x}$) was estimated from the slope of the plot of
6 cell density versus increased cell mass; the PMLA productivity was calculated
7 from the slope of the plot of PMLA concentration versus the production time.

8 **3. Result and discussion**

9 **3.1 Unravelling the effect of CaCO_3 addition on PMLA production**

10 The effect of CaCO_3 addition on cell growth and PMLA production is shown
11 in Fig.1. Without CaCO_3 addition, the pH of the broth dropped to 3.99 because of
12 the PMLA production. When CaCO_3 was added in the broth, culture pH of the
13 cultivations kept between 6.0 and 6.5. The highest PMLA production was
14 obtained at 1.5% CaCO_3 addition, while at above this CaCO_3 concentration,
15 PMLA production decreased. Moreover, it was found that with addition of CaCO_3 ,
16 the biomass slightly increased and then leveled off when the content of CaCO_3
17 was higher than 3.0%. In addition, our previous work had proved that pH around
18 6.0 was favorable for PMLA production [18]. Therefore, exogenous CO_3^{2-} and
19 Ca^{2+} may be the other two possible reasons for the positive effect of CaCO_3
20 addition on PMLA production. The negative effect of excessive CaCO_3 addition
21 might be attributed to the interaction between cells and CaCO_3 , which had a
22 detrimental effect on the rate of oxygen transfer, since DO was a key factor to
23 PMLA production [18]. However, the positive effect of CaCO_3 addition on
24 PMLA production were hard to expound according to the results in flasks,
25 because we could not distinguish the effects of pH, Ca^{2+} , and CO_3^{2-} on PMLA
26 production in flask. To clarify whether the exogenous CO_3^{2-} and Ca^{2+} were

1 responsible for the positive effect on PMLA production, the following
2 investigations would firstly focus on the effect of CO_3^{2-} on PMLA production in a
3 7.5 L bioreactor.

4 **3.1.1 Effect of CO_3^{2-} on PMLA production**

5 To clarify the effect of CO_3^{2-} on PMLA production, different soluble
6 neutralizers were used for PMLA production by keeping culture pH at 6.0 in
7 bioreactor. When NaOH or Na_2CO_3 was used as the neutralizer (Fig. 2a and b),
8 we found that there was no significant difference in PMLA production and cell
9 growth at the end of culture (the glucose was depleted), and the highest PMLA
10 productivity and $Y_{p/s}$ (g PMLA/g glucose), $0.74 \text{ g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ and 0.23 g/g respectively,
11 were obtained. However, compared with NaOH or Na_2CO_3 , the neutralizer
12 $\text{NH}_3 \cdot \text{H}_2\text{O}$ increased biomass by 23.4% but decreased PMLA concentration by
13 19.96%, because the presence of NH_4^+ in the broth served as an extra nitrogen
14 source which could promote cells growth and resulted in less carbon metabolism
15 for PMLA production. For NH_4^+ assimilation, *A. pullulans* possessed both
16 Nicotinamide adenine dinucleotide (NAD^+)-dependent glutamate dehydrogenase
17 (EC 1.2.1.2) and glutamine synthetase (EC 6.2.1.2)/ glutamate synthase (GOGAT)
18 (EC 1.2.7.1) pathway [21]. The above results showed that CO_3^{2-} was not the main
19 factor responsible for the positive effects of CaCO_3 on PMLA production. Thus,
20 the effect of Ca^{2+} on PMLA production in a 7.5 L bioreactor was investigated in
21 the following study.

22 **3.1.2 Effect of Ca^{2+} on PMLA production**

23 Fig. 3 shows the effect of exogenous Ca^{2+} concentration on PMLA production
24 with NaOH as the neutralizer in the 7.5 L bioreactor. Significant increase in
25 PMLA production by adding exogenous Ca^{2+} in the broth was observed after 48 h
26 cultivation. However, when CaCl_2 concentration was higher than 0.1 g/L , there

1 was no further increase in PMLA concentration. The maximum PMLA
2 concentration, up to 51.2 g/L with a productivity of $0.85 \text{ g} \cdot \text{h}^{-1} \cdot \text{L}^{-1}$ was obtained at
3 0.1 g/L CaCl_2 after 60 h cultivation. In contrast to other insoluble calcium salts of
4 organic acids [22], PMLA-Ca could be easily dissolved in water, even at a high
5 concentration up to 80 g/L [9, 23]. Thus PMLA-Ca could not act as a disinhibition
6 agent in the process of PMLA production. Adding exogenous Ca^{2+} could increase
7 PMLA production possibly due to morphological change of *A. pullulans*. For *A.*
8 *pullulans*, and the exogenous Ca^{2+} could increase yeast-like cell formation, thus
9 reducing the transition of yeast cells to mycelia [20]. It is worth mentioning that
10 the yeast-like form *A. pullulans* was the main PMLA producer [18]. In addition,
11 with increase of calcium concentration [24, 25], the malate formation increased
12 and the L-malate was the immediate precursor of polymerization for PMLA
13 production [5, 26, 27]. Therefore, it could be concluded that the Ca^{2+} contributed
14 to the improvement for PMLA production at constant pH conditions, and the pH
15 and Ca^{2+} were the two factors for promoting the PMLA concentration by CaCO_3
16 addition. Moreover, given the fact that the cost of Na_2CO_3 was lower than NaOH,
17 Na_2CO_3 was a better substitute for CaCO_3 as a neutralizer in the bioreactor for
18 PMLA production. Thus, in the following studies, 0.1 g/L CaCl_2 would be added
19 into the broth with Na_2CO_3 as the neutralizer for PMLA production except stating
20 elsewhere. Furthermore, the hypothesis that neutralizers and Ca^{2+} increase PMLA
21 M_w would be proved.

22 **3.2 Variation of PMLA M_w in the batch culture**

23 **3.2.1 PMLA M_w in the batch culture with Ca^{2+} addition and Na_2CO_3 as the** 24 **neutralizer**

25 After examining the main factors influencing PMLA production with the
26 addition of CaCO_3 , variation of PMLA M_w in the batch culture with the strain ipe-

1 were investigated at the same conditions as those in Fig.3 exception for CaCl_2
2 concentration (0.1 g/L) and neutralizer type (10% Na_2CO_3). The weight average
3 molecular weight (M_w), number average molecular weight (M_n), molecular weight
4 distribution (M_w/M_n), and other parameters of the produced PMLA measured at
5 the end of the culture are shown in Table 1. Regarding the molecular weights, gel
6 permeation chromatography analysis showed that there were two PMLA fractions
7 obtained at the end of the culture (Fig.4 and Table 1). They were M_w 172020Da
8 and M_w 10531Da, respectively, while 99.2629% of them were the latter. In these
9 conditions, M_w/M_n was 1.13 on average. The M_w of PMLA produced by the strain
10 ipe-1 was close to that by *Aureobasidium* sp. A-91[14] (Table 4).

11 The variation of PMLA molecular weight in the process of the batch culture is
12 shown in Fig.5b. It can be seen that the M_w increased in the first 48 h and then
13 decreased sharply, while the M_w was almost constant in the last 12 h of the culture
14 (from 60 h to 72 h). The variation of M_w was almost the same as that of $Y_{p/x}$ in
15 Fig.5a at the culture time from 0 to 60 h, while the differences appeared in the last
16 12 h, in which $Y_{p/x}$ decreased sharply because there was no carbon source for cell
17 growth. The PMLA with considerably high molecular weight (M_w =18.693 kDa,
18 M_n =11.276 kDa) was obtained at 48 h when $Y_{p/x}$ reached the highest point.
19 Therefore, it might be a good choice to achieve high M_w by increasing $Y_{p/x}$, while
20 it was prerequisite to maintain cell growth. Compared to the addition of NaOH or
21 Na_2CO_3 , the M_w was decreased by about 11% when $\text{NH}_3 \cdot \text{H}_2\text{O}$ was used as the
22 neutralizer (Table 2). When 0.1 g/L CaCl_2 was added in the broth, M_w was
23 increased by 13.5% and 26.3% when using NaOH and Na_2CO_3 as the neutralizers,
24 respectively. However, it is still unclear why Ca^{2+} addition with Na_2CO_3 as the
25 neutralizer could enhance PMLA M_w , which would be further discussed in the
26 next study.

1 **3.2.2 Mechanism discussion**

2 Melanin (including cellular and extracellular melanin) and
3 exopolysaccharide were two main by-products during PMLA biosynthesis [14,
4 15], and the reducing capacity (RC) activity of supernatant (i.e. a dose dependent
5 capacity in reducing hexocyanoferrate from the trivalent to divalent form)
6 significantly affected the synthesis of exopolysaccharide and melanin [17], which
7 probably resulted in variation in PMLA production. Since PMLA M_w increased
8 from 24h to 48h, thus we focused on the variations of melanin, exopolysaccharide
9 and RC activity between 24 h and 48 h. Firstly, melanin and RC activity were
10 monitored during the fermentations with different neutralizers (without Ca^{2+}
11 addition). As shown in Fig. 6a, the RC activity with NaOH as neutralizer was
12 lower than that with Na_2CO_3 , and meanwhile a large amount of extracellular
13 melanin was produced (Fig.6b) when NaOH was used as the neutralizer. The
14 extracellular melanin release was related with RC activity in the broth because the
15 melanin was produced for protecting the cells from damage by the oxidizing force
16 of the broth [28]. Therefore, when the RC activity was lower, more extracellular
17 melanin would be produced in order to alleviate the negative effect of decreasing
18 RC activity on cells. However, such variation produced a negligible effect on
19 PLMA M_w and production (Table 2). Thus, it could be concluded that RC activity
20 and extracellular melanin did not affect PMLA M_w .

21 However, when Ca^{2+} was added into the broth, the cellular melanin greatly
22 increased when NaOH or Na_2CO_3 was used as a neutralizer (Fig.6c), while the
23 extracellular melanin was much lower than that without Ca^{2+} addition (Fig.6b).
24 This implied that adding Ca^{2+} could accelerate melanin deposition on cell walls,
25 thus decreasing the melanin release into the broth as black granulates. And at the
26 same time, compared with the case without Ca^{2+} addition, PMLA production and

1 M_w were increased, but biomass was decreased no matter NaOH or Na_2CO_3 was
2 used as the neutralizer with Ca^{2+} addition. It could be speculated that more
3 melanin depositing on cell walls would inhibit the cell growth and their metabolic
4 capacity on PMLA. Therefore, the addition of Ca^{2+} could increase cellular
5 melanin, and the higher cellular melanin decreased the biomass, which resulted in
6 an increase of PMLA M_w .

7 **3.3 Enhancement of PMLA M_w with Ca^{2+} added repeated batch culture**

8 The above results showed that $Y_{p/x}$ (g PMLA/ g biomass) began to decrease
9 from 48 h and the produced high M_w was depolymerized at the same time (Fig.5).
10 And when glucose was depleted after 60 h, biomass still had a slight increase at
11 the cost of PMLA concentration (Fig.3). Therefore, in order to enhance M_w ,
12 repeated batch culture was further performed to maintain cell in growth phase
13 before glucose was depleted, and the produced PMLA was removed in order to
14 avoid its depolymerization. When initial batch fermentation arrived at the end of
15 exponential growth, 3 L of the broth was removed, and another 3 L of the fresh
16 medium was fed into the bioreactor. The fermentation was restarted under the
17 same conditions as the batch fermentation. The results of three cycles with *A.*
18 *pullulans* ipe-1 were showed in Table 3 and Fig.7, where 57.07 g/L of PMLA
19 concentration on average was obtained with a productivity of $1.1\text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$.
20 Compared to the batch culture, the average M_w in the three cycles increased by
21 84.4%, reaching 19.51 kDa. Therefore, M_w of PMLA could be highly enhanced
22 by calcium ion added repeated batch culture. There are two possible reasons for
23 this phenomenon. On the one hand, adding exogenous Ca^{2+} in the broth could
24 increase cellular melanin, and the higher cellular melanin might decrease biomass,
25 which resulted in an increase of PMLA M_w . On the other hand, the produced
26 PMLA was bled before the depolymerization by the PMLA hydrolase in the

1 repeated batch culture, thus keeping their high M_w for the subsequent downstream
2 purification. For *P. polycephalum* in batch culture, Holler et al. [29] also reported
3 that the plasmodia had a maximum intracellular level of PMLA concentration
4 when they grew at maximum rate. It was found that the depolymerization of
5 PMLA occurred in the culture of *P. polycephalum* after cell growth [30], and
6 PMLA hydrolase was responsible for this depolymerization [31]. Then Gasslmaier
7 and Holler [32] reported that the catalytic mechanism of the hydrolase dominated
8 the substrate-binding specificity and the depolymerization proceeded from the
9 hydroxy-terminus towards the carboxyterminus, thereby degrading the polymer to
10 L-malate.

11 **4. Conclusion**

12 In β -poly(L-malic acid) production by *A. pullulans* ipe-1, the Ca^{2+} addition
13 contributed to the improvement of PMLA production at constant pH conditions,
14 and the pH and Ca^{2+} were the two factors for promoting the PMLA concentration
15 by $CaCO_3$ addition. Further, adding exogenous Ca^{2+} in the broth of batch culture
16 could enhance M_w of PMLA, and the molecular weight of PMLA increased from
17 12.522 to 18.693 kDa and then decreased in the late exponential growth phase.
18 The M_w varied with $Y_{p/x}$ (g PMLA/g biomass), and the maximum M_w was
19 achieved when $Y_{p/x}$ reached its maximum point. In Ca^{2+} added repeated batch
20 culture, the produced PMLA was bled before its depolymerization, thus keeping
21 their high M_w for the subsequent downstream purification. Compared with the
22 results in batch culture, M_w increased by 84.4% (up to 19.51kDa) with a
23 productivity of $1.1 \text{ g} \cdot \text{h}^{-1} \cdot \text{L}^{-1}$ during Ca^{2+} added repeated batch culture. Thus, the
24 present work not only provides an efficient approach to increase PMLA
25 productivity, but also obtains superior quality PMLA product with high M_w .

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Figures

Fig. 1 Effect of CaCO_3 addition on PMLA production in flasks. Cultivations were conducted in 500 mL indented conical flasks containing 50 mL flasks fermentation medium at initial pH 4.5 (25 °C) as described in the text. PMLA (■), Biomass (□), pH (▼). Error bars in the figure represent standard deviations from three individual experiments.

Fig. 2 Effect of neutralizing agents (a) NaOH, (b) Na₂CO₃ and (c) NH₃·H₂O on cell growth and PMLA production. PMLA (■), Biomass (□), Glucose (▲). Error bars in the figure represent standard deviations from two individual experiments.

Fig. 3 Effects of CaCl_2 on (a) PMLA production, (b) biomass and (c) glucose with NaOH as the neutralizer. The initial CaCl_2 concentrations (g/L) were 0 (●), 0.04 (Δ), 0.1(■), 0.5(▲), 1 (○). Error bars in the figure represent standard deviations from two individual experiments.

4 **Fig. 4** The GPC chromatogram of ipe-1 strain's product (a), HPLC chromatogram of
 5 the hydrolysate of ipe-1 strain's product (b), and HPLC chromatogram of the control
 6 L-malic acid (c). The peaks in Fig.4a at t_R 9.8min and t_R 12.2min were belonged to the
 7 solvent peaks.

Fig. 5 Results of kinetic analysis for PMLA fermentations at 0.1 g/L CaCl_2 . Productivity of PMLA (Δ) and Biomass ($\circ$), Glucose consumption rate ($\bullet$), $Y_{p/s}$ (∇), $Y_{p/x}$ ($\blacksquare$), $Y_{x/s}$ ($\blacktriangle$), M_w ($\blacktriangledown$), M_w/M_n ($\square$). The molecular weight was relative to polyethylene glycol standards. Error bars in the figure represent standard deviations from two individual experiments.

Fig.6 Effects of neutralizing agents on (a) reducing capacity (RC) and (b, c) melamin. Na₂CO₃ (○), NaOH (■), NH₃·H₂O (▲), Na₂CO₃ with 0.1 g/L CaCl₂ (●), NaOH with 0.1 g/L CaCl₂ (□). Error bars in the figure represent standard deviations from two individual experiments.

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7 Fig.7 PMLA production with calcium ion added repeated batch culture. PMLA (■),

8 Biomass (□), M_w (▼).

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