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

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ABSTRACT

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

1. Introduction

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

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

Although the available method for increase of PMLA M_w cannot be found in the present literature yet, Nagata et al. [14] reported that the addition of $CaCO_3$ to culture media could increase the PMLA concentration, and the M_w of obtained PLMA was relatively high among the known results. Inspired by this, we hypothesize that $CaCO_3$ addition during the fermentation can also increase the PLMA M_w. However, the addition of $CaCO_3$ would produce various effects on the PLMA fermentation. For instance, when the experiment was carried out in flasks, the addition of $CaCO_3$ would change both ionic

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strength and pH during the fermentation. When producing PMLA in bioreactor, the addition of $CaCO_3$ as neutralizer could result in a highly viscous suspension because the cells interacted with the precipitates, and this would have a detrimental effect on the rate of oxygen transfer. Therefore, it is difficult to clarify the mechanisms how $CaCO_3$ addition did affect the PMLA production and to find suitable fermentation conditions to regulate the M_w of PMLA products. To the best of our knowledge, the variation of PMLA M_w during the batch culture and the strategies to enhance PMLA M_w were not investigated yet.

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

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 compositions (w/v) of the seed culture medium were as follows: 8% glucose, 0.2% $NaNO_3$, 0.01% KH_2PO_4, 0.02% $MgSO_4{\cdot}7H_2O$, 0.05% KCl and 0.1% tryptone (LP0042, Oxoid LTD., Basingstoke Hampshire, England) in deionized water. The compositions of the fermentation medium in flasks were the same as the seed culture except for the $CaCO_3$ concentration. For the production of PMLA in the 7.5L bioreactor (BioFio®110, New Brunswick Scientific, USA), the following medium was used (w/v): 19% glucose, 1.5% tryptone, 0.6% $NaNO_3$, 0.5% yeast extract (LP0021, Oxoid LTD., Basingstoke Hampshire, England), 0.05% KH_2PO_4, 0.02% $MgSO_4{\cdot}7H_2O$, 0.05% KCl and 5 ppm $ZnSO_4{\cdot}7H_2O$ in deionized water.

2.3. Culture method

In all fermentation experiments, 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 2d on the rotary shaker (HYG-A, Taicang Experimental Equipment Factory, China) at 180 rpm. For flask culture, a 500 mL flask containing 50 mL medium was inoculated with 5 mL of seed culture broth and cultured at 25 °C for 5 d on the rotary shaker (180 rpm). The seed prepared for bioreactor was cultured in six Erlenmeyer flasks each time, respectively. After culturing and mixing the seed culture broth, 400 mL of the mixture was transferred to the 7.5 L bioreactor containing 4 L of the fermentation culture medium. The temperature, aeration rate and pH in the bioreactor was kept at 25 °C, 0.25–2.5 vvm and 6.0, respectively, and the pH was kept constant automatically by adding alkali or 1 M H_2SO_4. DO was controlled by agitation and detected by a DO electrode. When DO dropped to the set value of 70%, the agitation rate was automatically increased based on the value of DO concentration. For repeated-batch fermentation, when initial batch fermentation arrived at the end of exponential growth, 3 L of the broth was removed, and 3 L of the fresh medium was feed into the bioreactor. The fermentation was restarted under the same conditions as the batch fermentation. All fermentation experiments were performed at least twice, to ensure the observed trends were correct and reproducible. Not all the data was presented in the figures.

2.4. Analytical methods

The culture broth (8 mL) was centrifuged in a high speed centrifuge (4–16 K, Sigma, Germany) at 10,000 × *g* for 8 min and the resulting supernatants were used for the measurements of PMLA, glucose and absorbance. For the measurement of biomass, the cells were washed three times with 8 mL distilled water, dried to constant weight at 90 °C. However, excess $CaCO_3$ was removed by the addition of 1 M HCl before the measurement of biomass in flask conditions. 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 (LC20AT, Shimadzu, Japan) equipped with a SPR-H column (Shimadzu, Japan). The separation was performed at UV 210 nm, a temperature of 40 °C, a flow rate of 0.6 mL/min and 4 mM perchloric acid was used as eluant. Glucose concentrations of the supernatants were measured by a biosensor with glucose oxide electrodes (SBA-40C, Biology Institute of Shandong Academy of Sciences, China). To recover PMLA from broth, the procedures were carried out as described in Nagata et al. [14]. The supernatant (5 mL, pH6.0) was warmed to about 50 °C and MeOH (1.8 mL) was added dropwise with stirring to the warmed supernatant. A resulting string precipitate of polysaccharide was filtrated with 0.2 μm filter. MeOH (10 mL) was further added dropwise with stirring to the mixture. The mixture was left 12 h with mild stirring at 4 °C. A resulting precipitate was collected by filtration, suspended in deionized water (3 mL) and fully stirred. Small amounts of insoluble impurities were removed by filtration. MeOH (6 mL) was added dropwise under stirring to the filtrate, and then the mixture was left for 12 h at 4 °C. A resulting precipitate was collected by filtration and washed successively with 60% MeOH. The pulverized and air-dried precipitate was used in subsequent experiments. The PMLA M_w was evaluated by gel-permeation chromatography using Waters apparatus with TSKgel G3000PW$_{XL}$ (Tosoh, Tokyo), which was calibrated with polyethylene glycol standards (Tosoh, Tokyo and Polysciences, Inc., Warrington) under the following conditions: flow rate, 1.0 mL/min; eluate, 0.2 M sodium phosphate buffer, pH6.8; detector RI. Melanin was measured as described in Gadd et al. [16]. To determine cellular melanin, washed cell pellets, obtained by centrifuging 5 mL culture samples at 4000 × *g* for 5 min, were autoclaved with 3 mL of 1 M NaOH (20 min, 120 °C). After cooling and centrifugation, 3 mL of borate buffer (pH 8.0) was added to the supernatant and the absorbance at 540 nm was measured. Extracellular melanin was determined by acidifying the cell-free culture supernatants to pH 2.0 with 1 M HCl and centrifuging at 10,000 × *g* for 10 min. Then extracellular melanin was extracted with 1 M NaOH and the absorbance at 540 nm was measured as described previously. Extracellular polysaccharide was isolated as described in Nagata et al. [14]. The reducing capacities (RC) of culture filtrates were determined by ferri-cyanide reduction method and calculated with the formula: RC = [(A_{420} control − A_{420} sample)/A_{420} control] × 100% [17].

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