Differences between *Aureobasidium Pullulans* CGMCC No.3337 and *Aureobasidium Pullulans* TKPM10017 on Synthesis of Poly (β-L-malic-acid) Metabolism

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Keywords: Poly (β-L-malic-acid) (PMLA), *Aureobasidium pullulans*, Protein spectrum experiment, Intracellular enzyme activity.

Abstract. Poly (β-L-malic-acid) (PMLA) is a sort of polyester and absolute biodegradable material. PMLA is a new type of nontoxic macromolecule material and with favorable biodegradation and biocompatibility. PMLA has extensive applications in the areas of pharmacy and medical industry. Application foreground of PMLA is very extensive. Poly malic acid has widely attracted the attention of people, but the studies of poly malic acid - *Aureobasidium pullulans* (*A.pullulans*) and the metabolic mechanism of *A.pullulans* is rarely reported, the researchers abroad studied *A.pullulans* is earlier than domestic researchers. The Key Laboratory of Industrial Microbiology, Ministry of Education, Tianjin University of Science and Technology preserve two strains which have different production--*Aureobasidium pullulans* CGMCC No.3337 and *Aureobasidium pullulans* TKPM10017. *A.pullulans* CGMCC No.3337 was selected from *A.pullulans* TKPM10017 by UV and He-Ne laser. There were significant differences in production of PMLA. In order to study the significant differences, we carried out the protein spectrum experiment the extraction of genome of *A.pullulans* TKPM10017 and *A.pullulans* CGMCC No.3337 and the Polymerase Chain Reaction of ATP synthase alpha subunit and ATP synthase beta subunit and gene sequence analysis. From the metabolic pathway perspective analyze on the causes of such differences.

Introduction

Poly (β-L-malic-acid) (PMLA) is a functional aliphatic polyester which is composed of malic acid repeating units [1]. Having pendant carboxylic groups allows the

introduction of functional groups and molecules, including drugs via chemical modification. These advantages make it important to investigate high purity, high-yield production for this polymer. PMLA is a new type of nontoxic macromolecular material with favorable biodegradation and biocompatibility [2, 3] , which has gained great industrial interest for its potential applications in medicine[4] and other industries. For example, it was reported that a PMLA-based drug delivery system and biodegradable nano particles could be used for tumor targeting [5]. Therefore PMLA can be used as prodrug or for drug delivery system, which has attracted a great deal of interest in industries [6, 7].

PMLA can be obtained by the method of chemical synthesis or microbial fermentation. Recently, biosynthesis has been extensively investigated. Schimada, K firstly reported biosynthetic PMLA by Penicillium cyclopium. Since then Physarum polycephalum, Aureobasidium sp., Aureobasidium pullulans [8, 9], and several other myxomycetes and mitosporic fungi [10] have been reported to produce the polymer successively. According to the reports that Aureobasidium sp. is the bestest strain to product PMLA, yield changes following the strains of the different properties.However, under natural conditions, the productivity of PMLA is still in a low level, and few domestic researches on the production of PMLA by Aureobasidium pullulans have been carried out [11, 12].

Aureobasidium pullulans CGMCC No.3337 is the high-yielding strain, and its production of PMLA is much higher than *Aureobasidium pullulans* TKPM10017 (the original strain of the high-yielding strain). Such a giant change attracts our attention to investigate. We have known that there are obvious differences in production of PMLA, while we can't get across the reason. We try to make the reason clear through the metabolic flux analysis and metabonomics analysis.

Materials and Methods

Strain and Medium

Aureobasidium pullulans CGMCC No.3337 was isolated by the Key Laboratory of Industrial Microbiology, Ministry of Education, Tianjin University of Science and Technology. *A.pullulans* CGMCC No.3337 was selected from *A.pullulans* TKPM10017 by UV and He-Ne laser. The cells were grown on LB mediums for 3 days at 25 ℃ and stored at 4 ℃.

The medium for inoculum preparation (seed medium) contained the following components (g/L) : glucose 140 , yeast extract 3 , $(NH_4)_2SO_4$ 1 , succinic acid 2 , K_2CO_3 0.4 , KH_2PO_4 0.1 , $CaCO_3$ 20, $ZnSO_4 \cdot 7H_2O$ 5×10^{-3} , $MgSO_4 \cdot 7H_2O$ 0.1, corn steep liquor 0.1%(v/v).

The fermentation medium for flask culture was consisted of the following components (g/L): glucose 180, peptone 35, KCl 0.5, KH_2PO_4 0.1, $NaNO_3$ 2, $MnSO_4$ 0.05, $MgSO_4 \cdot 7H_2O$ 0.3, $CaCO_3$ 20. The initial pH of the medium was adjusted by using 5 M NaOH and 6 M HCl to 7.0.

Cultivation Methods

Shake-flask Culture. The cells growing on newly prepared were transferred to 500

ml shake flask containing 50 ml seed medium and incubated at 25 ℃ and 200 rpm for inoculums preparation. The cells used for experiment was carried out by 500 ml shake flask containing 50 ml fermentation medium and incubated at 25 ℃ and 200 rpm.

Fermentation Cylinder Culture. The seed prepared for the bioreactor was cultured in the Erlenmeyer flask each time. When its absorbance measured at 600 nm using a ultraviolet spectrophotometer (UV-1800PC, China), was at 0.6-0.7, transferred it to a 10.0 L bioreactor (Shanghai, China) containing 7 L fermentation culture medium. Fermentation conditions: the dissolved oxygen 30%, ventilation rate 1:1.2, initial stirring speed 300-600 rpm, pH 7.0, fermentation temperature 25 ℃, inoculum size 10%,fermentation time 7 days.

OD and Biomass

OD. Cell growth was determined by measuring the absorbance at 600 nm using a ultraviolet spectrophotometer (UV-1800PC, China).

Biomass Concentration. Two milliliter of broth mixed 3 M HCl (aim to wipe off calcium carbonate) was centrifuged at 2000xg at 4 ℃ for 20 min (Microfuge 18, Beckman Coulter Co., Fullerton, CA). Then, the precipitate were washed twice with distilled water and centrifuged again to remove impurities. After the precipitate were dried at 80 ℃ overnight (>8h), dry weight amortized computation with fermentation broth was determined as biomass.

Quantitative Analysis of PMLA

The concentration of PMLA was determined by a reversed phase high performance liquid chromatography method on a column of prevail C18 (4.6 mm×250 mm, 4 μm) with a mobile phase containing acetonitrile and 0.025 M KH_2PO_4 buffer (pH 2.5) (5:95, v: v), the flow rate of mobile phase was 1.0 mL/min, the detection wavelength was 210 nm and the column temperature was 25 ℃. Before sampling, the fermentation broth was centrifuged and hydrolysed. L-malic acid were completely separated and determined in 4 minutes.

Protein Spectrum Experiment

The experimental method: protease digestion, LC-MS/MS detection. HPLC conditions: mobile phase: liquor A H_2O (mass spectrometry), liquor B acetonitrile (mass spectrometry); Flow rate: 200 ul/min (after shunt 2 ul/min); the elution gradient: 120min (5%B to 35%B in 40 min, to 95% in 20 min, balance for 20min); Sample: sample automatically; Sample quantity: 20 ul.

The Detection of Intracellular Enzyme Activity

We detect the intracellular enzymes: Pyruvate carboxylation kinase enol phosphate, Pyruvate carboxylase, Malate synthetase Malic dehydrogenase, Fumarate hydratase [13, 14].

Results

The Production of PMLA from *A.pullulans* CGMCC No.3337 and *A.pullulans* TKPM10017

As is shown in the table 1, the maximum PMLA concentration(P max),the optimal specific product yield(YP/X) and the optimal product yield(YP/S) of *A.pullulans* CGMCC No.3337 are much higher than *A.pullulans* TKPM10017, the specific growth rate(μ), the optimal cell production rate(QX), the optimal production formation rate(QP), the maximum cell density(X max), the optimal cell yield(YX/S) of *A.pullulans* CGMCC No.3337 are respectively a little bit higher than *A.pullulans* TKPM10017. The results showed that the growth conditions of *A.pullulans* CGMCC No.3337 are better than *A.pullulans* TKPM10017. Through mutagenesis, the maximum PMLA production of *A.pullulans* CGMCC No.3337 could reach 78.85±0.329 g/L, which was increased by 85.14 % compared with *A.pullulans* TKPM10017.As is shown in Fig. 1.

Table1 The growth condition of A.pullulans and yield in the process of fermentation

	μ (h^{-1})	Q_X(g/Lh)	Q_P(g/Lh)	X_{max}(g/L)	P_{max}(g/L)	$Y_{X/S}$(g/g)	$Y_{P/S}$(g/g)	$Y_{P/X}$(g/g)
A.pullulans TKPM10017	0.1387	0.7867	0.6825	43.1	42.59	0.2395	0.2199	0.9186
A.pullulans CGMCC No.3337	0.1579	0.9142	0.9575	49.23	78.85	0.2735	0.4583	1.6758

μ-the specific growth rate; Q_X-the optimal cell production rate; Q_P- the optimal production formation rate; X_{max}-the maximum cell density; P_{max}-the maximum PMLA concentration; $Y_{X/S}$-the optimal cell yield; $Y_{P/S}$-the optimal product yield; $Y_{P/X}$-the optimal specific product yield.

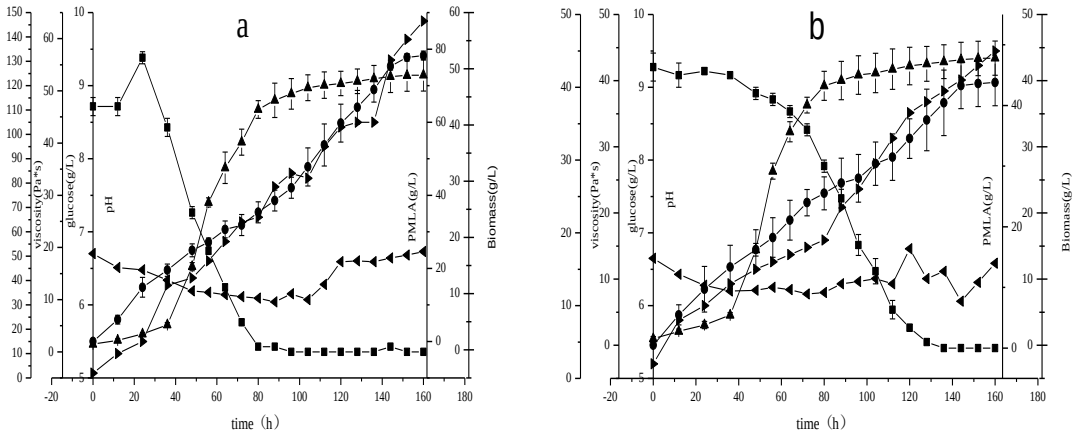

Figure 1. The differences of the strain *Aureobasidium pullulans* CGMCC No.3337 and the strain *Aureobasidium pullulans* TKPM10017 on the synthesis of poly(β-L-malic-acid) metabolism. *Aureobasidium pullulans* CGMCC No.3337(a), *Aureobasidium pullulans* TKPM10017(b), viscosity(►),the residual glucose(■),pH(◄),biomass(▲),PMLA(●).

Protein Spectrum Analysis Total Protein of Aureobasidium Pullulans

Protein spectrum according to the results: 51 protein were detected in *A.pullulans* TKPM10017; while, 55 protein were detected in *A.pullulans* CGMCC No.3337. Among the proteins, there are 8 protein showed increased over 40% in *A.pullulans* CGMCC No.3337 than in *A.pullulans* TKPM10017; while there are 12 protein showed decreased over 40% in *A.pullulans* CGMCC No.3337 than in *A.pullulans* TKPM10017.

The protein spectrum results show that many proteins and enzymes, such as ATP synthase subunit beta, UDP-glucose pyrophosphorylase, glucose-6-phosphate isomerase, pyruvate carboxylation kinase enol phosphate, malic dehydrogenase , 6-phosphofructokinase subunit beta, fumarate hydratase, pyruvic carboxylase, Fatty acid synthase subunit alpha, Protein kinase C-like 1, malate synthase, the proteins and enzymes acting the key enzymes respectively in Tricarboxylic acid cycle, Pyruvate carboxylation and Glyoxylate cycle play an important role. The results are shown in the following table 2.

Table 2. (a) Protein spectrum analysis total protein of *A.pullulans* TKPM10017

Accession	Coverage	# PSMs	# AAs	MW [kDa]	Score	Description
P00830	18.79	28	511	54.8	59.3	ATP synthase subunit beta, mitochondrial OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c)
P08417	8.03	16	488	53.2	83.2	fumarase FUM1 [Saccharomyces cerevisiae S288c]
P07251	8.81	8	545	58.6	63.9	UDP-glucose pyrophosphorylase
P10490	6.29	17	966	110.7	93.1	RecName: Full=Phosphoenolpyruvate carboxylase 1; Short=PEPC 1; Short=PEPCase 1
P11154	4.07	9	1,178	130.1	91.6	Pyruvate carboxylase 1 Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)
P12709	5.78	20	554	61.3	75.4	Glucose-6-phosphate isomerase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c)
P13697	6.13	5	572	64.0	89.3	RecName: Full=NADP-dependent malic enzyme; Short=NADP-ME; AltName: Full=Malic enzyme 1
P16862	2.09	4	959	104.6	76.0	6-phosphofructokinase subunit beta OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c)
P30952	4.23	10	554	62.8	86.5	malate synthase MLS1 [Saccharomyces cerevisiae S288c]

PSMs-Number of peptides; # AAs-Number of amino acids; MW [kDa]-molecular weight

Table 2. (b) Protein spectrum analysis total protein of A.pullulans CGMCC No.3337

Accession	Coverage	# PSMs	# AAs	MW [kDa]	Score	Description
P00830	19.77	51	511	54.8	83.6	ATP synthase subunit beta, mitochondrial OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c)
P08417	8.31	29	488	53.2	83.2	fumarase FUM1 [Saccharomyces cerevisiae S288c]
P10490	8.52	33	966	110.7	93.1	RecName:Full=Phosphoenolpyruvate carboxylase 1; Short=PEPC 1; Short=PEPCase 1
P11154	6.79	17	1,178	130.1	91.6	Pyruvate carboxylase 1 Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)
P13697	8.12	8	572	64.0	89.3	RecName: Full=NADP-dependent malic enzyme; Short=NADP-ME; AltName: Full=Malic enzyme 1
P19097	2.17	5	1887	206.8	30.8	Fatty acid synthase subunit alpha OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c)
P24583	3.65	10	1151	131.4	27.3	Protein kinase C-like 1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c)
P30952	5.83	11	554	62.8	86.5	malate synthase MLS1 [Saccharomyces cerevisiae S288c]

PSMs-Number of peptides; # AAs-Number of amino acids; MW [kDa]-molecular weight

The Detection of Intracellular Enzyme Activity

According to the formula $U/ml=\frac{\frac{\Delta A}{min} \text{ *the test volume*dilution ratio}}{\text{absorption coefficient*optical path*enzyme volume}}$ (the test volume: 100μL, dilution ratio: 1, absorption coefficient: $\varepsilon_{340}=6.22\times10^3$ L/(mol cm), optical path:3mm, enzyme volume:5μL)count the intracellular enzyme enzyme activity.The results reflected in Fig. 2.

Comprehensive analysis the above figures and the metabolic pathways of Aureobasidium pullulans, Pyruvate carboxylation kinase enol phosphate, Malate synthase, Malic dehydrogenase and Fumarate hydratase in the Tricarboxylic cycle, Pyruvic carboxylase in the Pyruvate carboxylation, Malate synthase, Malic dehydrogenase in the Glyoxylate cycle, the activity of the five kinds of intracellular enzyme in *A.pullulans* CGMCC No.3337 were significantly higher than *A.pullulans* TKPM10017. The test result of the experimental detection of intracellular enzyme activity confirms the results of the protein mass spectrometry experiments that the enzymes play an important role in the metabolic pathways. Therefore, these data can make a conclusion that the enzymes make a great effect to generate PMLA.

Synthesizing the results of the protein mass spectrometry experiments analysis total protein of Aureobasidium pullulans and the experimental detection of intracellular enzyme activity: the results of the protein mass spectrometry experiments analysis total protein of Aureobasidium pullulans indicates that the number of proteins and enzymes in *A.pullulans* CGMCC No.3337 are obviously more than *A.pullulans* TKPM10017; the results of the experimental detection of intracellular enzyme activity illustrates that the activity of the five kinds of intracellular enzyme in *A.pullulans* CGMCC No.3337 were significantly higher than *A.pullulans* TKPM10017. The results have shown that the increase of the number of enzymes enhance the enzyme activity, lead to the increase of metabolic flux, increase the production of PMLA.

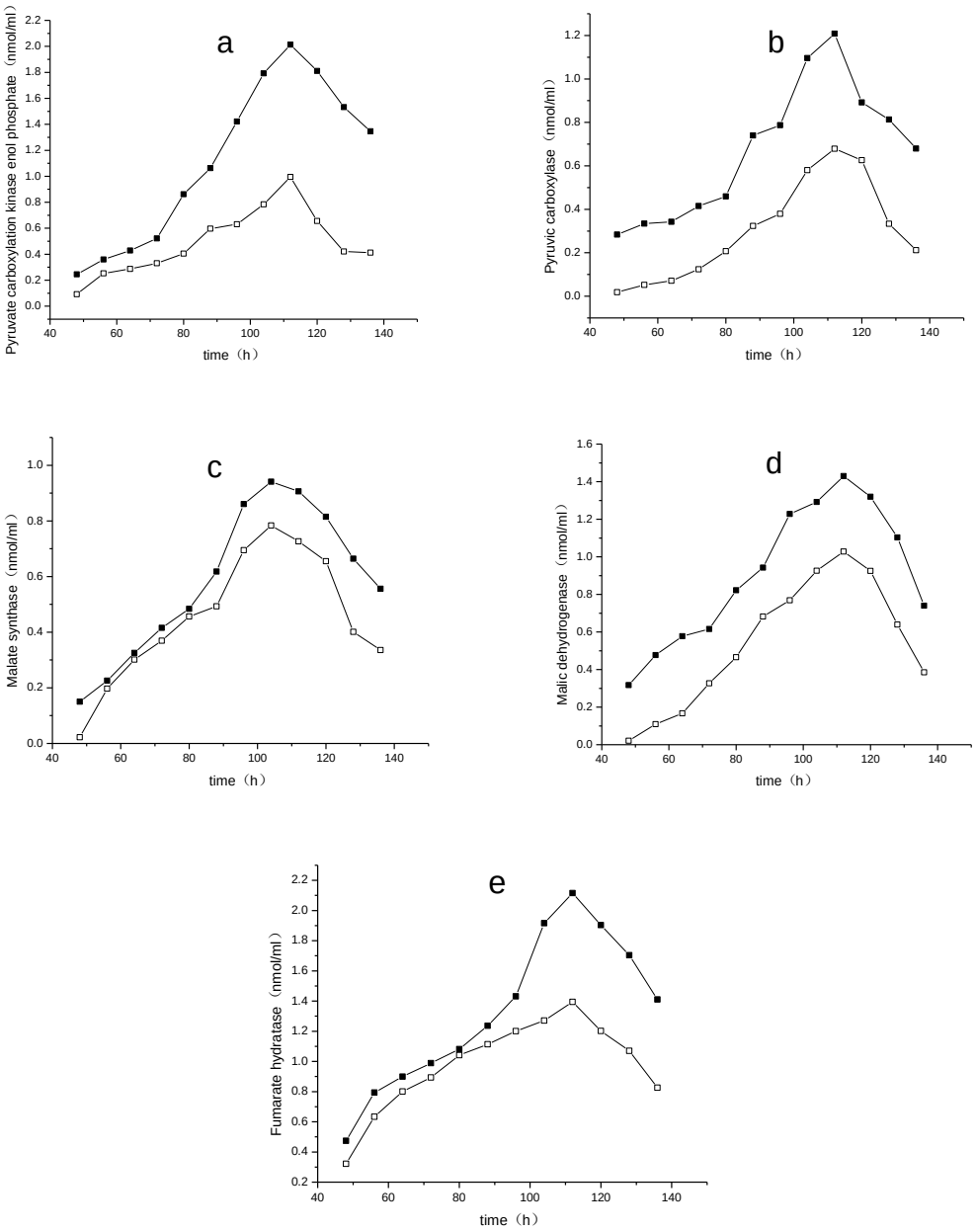

Figure 2. Pyruvate carboxylation kinase enol phosphate (a), Pyruvic carboxylase(b), Malate synthase(c), Malic dehydrogenase(d),Fumarate hydratase(e), enzyme activity change over time. *A.pullulans* TKPM10017 (□), *A.pullulans* CGMCC No.3337(■).

Discussion

The results of the protein spectrum analysis total protein of Aureobasidium pullulans indicates that the number of proteins and enzymes in *A.pullulans* CGMCC No.3337 are obviously more than those in *A.pullulans* TKPM10017;the results of the enzyme activity detection experiments illustrates that the activity of the five kinds of intracellular enzyme in *A.pullulans* CGMCC No.3337 is significantly higher than in *A.pullulans* TKPM10017.The results have shown that the increase in the number of enzymes enhances the enzyme activity, leading to the increase of metabolic flux, PMLA production.

The study has shown that there are significant differences of PMLA production from *A.pullulans* CGMCC No.3337 and *A.pullulans* TKPM10017. Through carrying on the protein mass spectrometry experiments analysis total protein of Aureobasidium pullulans and the experimental detection of intracellular enzyme activity, we have found out and verified the reason why the production of PMLA increased significantly from *A.pullulans* CGMCC No.3337. This new and improved theory of PMLA production process methodology will conceivably provide significant contribution and insight, and possibly also to other relevant fermentation processes for the improvement of product yield and productivity at the industrial scale.

Acknowledgement

This work was financially supported by the Program of Tianjin University of Science and Technology, and the Program of the company of Tianjin Peiyang Biotrans, and the Program of Tianjin science and technology plan under No.14ZCZDTG00025. The authors would like to thank these organizations for their generous financial support.

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