Comparative study of different molecular weight pullulan productions by *Aureobasidium pullulans* CGMCC No.11602

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Abstract

Pullulan productions by *Aureobasidium pullulans* CGMCC No.11602 were conducted using the initial culture (IC) medium and the optimized culture (OC) medium, respectively, in which pullulan with significantly different molecular weights was obtained. Under the IC medium condition, the pullulan molecular weight (M_w) reached 288,403 Da with a yield of 64.12 g/L after 96 h fermentation period. However, the pullulan molecular weight was much higher (M_w , 3,715,352 Da), while the yield of pullulan was lower (40.12 g/L) using the OC medium. The FTIR spectra indicated that pullulan produced using the IC and OC medium were similar. Transcriptome analysis showed that a total of 871 differentially expressed genes (DEGs) were screened and “N-glycan biosynthesis” and “other types of O-glycan biosynthesis” were the most highly represented differential metabolic pathways (DMPs). Specifically, the genes involved in the two DMPs consistently pointed to glucosyltransferase genes (*GTF*), all of which were up-regulated in the OC medium when compared with those in the IC medium. Further studies showed that the activity and the relative quantity (RQ) of *GTF* transcription were remarkable higher, which were coincident with the slower decrease in the molecular weight of pullulan in the OC medium than those in the IC medium during the late stage of fermentation. The results indicated that *GTF* may be involved in the regulation of pullulan molecular weight.

Keywords Pullulan · Molecular weight · Glucosyltransferase · Transcriptome analysis · qRT-PCR

Introduction

Pullulan is an important exopolysaccharide which has numerous applications in several industrial sectors such as pharmaceutical, food and cosmetic industries (Wang et al. 2015a; Kou et al. 2014). This macromolecule is produced by yeast-like fungus *A. pullulans* and has unique linkage patterns of repeating units of α -1,4 and α -1,6 glucans, which endows pullulan with structural flexibility and superior solubility (Li et al. 2015a; Prasongsuk et al. 2018). The M_w of

pullulan ranges from 1.5×10^4 to 1.0×10^7 depending on the culture condition and strains used (Kim et al. 2000). Compared with the other polysaccharides, pullulan has better biocompatibility owing to single substance composition and aggregation form (Li et al. 2015b) and thus the application research of pullulan in the biomedical field has become a research hotspot in recent years (Amrita et al. 2015; Bulman et al. 2015; Singh et al. 2017). On the other hand, current research results showed that the bioactivity of polysaccharides was significantly influenced by average M_w and the molecular weight distribution. In principle, the biocompatibility becomes well when the polysaccharides have larger molecular weight and narrower molecular weight distribution (Chan et al. 2015). Thus, pullulan with high M_w has higher value and broader application scope (Liu et al. 2017).

Pullulan fermentative production with *A. pullulans* had been widely studied since the 1970s. Researchers have made many efforts to optimize nutrients and environmental conditions (Wu et al. 2012), mainly aimed at increasing pullulan production. The effects of carbon source, shear stress, agitation, aeration and dissolved oxygen levels, production enhancers (such as zinc and iron), and cell morphology

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have been studied to improve pullulan production (Cheng et al. 2011a). However, much fewer studies have focused on the regulation of pullulan molecular weight compared with pullulan yield optimization studies. Most researchers have concluded that the molecular weight of pullulan constantly changed and decreased at the later stages of fermentation owing to pullulanase and amylase in the fermentation broth (Cheng et al. 2011b). Moreover, few scholars have studied the effect of pullulan synthetases on the molecular weight of pullulan.

In this study, pullulan production by *Aureobasidium pullulans* CGMCC No. 11602 was conducted using the initial culture (IC) medium and the optimized culture (OC) medium, respectively, in which pullulan with significantly different molecular weights was obtained. Transcriptome analysis was used to identify the key factors which influenced the molecular weight of pullulan. Further, the qRT-PCR method and enzyme activities in vitro test were used to evaluate the role of *GTF* in pullulan biosynthesis regulation.

Materials and methods

Microorganism and media

A. pullulans (CGMCC No. 11602), which produced pullulan with high yield and no pigment, was obtained through our laboratory breeding and was maintained at 4 °C on potato dextrose agar (PDA) slants and sub-cultured every 2 weeks. For long-term storage, cultures were maintained at − 80 °C in a 20% glycerol solution.

The seed medium (initial pH of 6.5) was as follows (g/L): glucose, 50; yeast extract, 2.5; (NH₄)₂SO₄, 0.6; K₂HPO₄, 5.0; NaCl, 1.0; and MgSO₄·7H₂O, 0.2. The seeds were incubated at 28 °C and 230 rpm on a shaker for 36 h. The initial culture (IC) medium contained the following components (g/L): sucrose, 100; yeast extract, 1.7 g, (NH₄)₂SO₄, 0.6; K₂HPO₄, 5.0; NaCl, 1.0; and MgSO₄·7H₂O, 0.2; The optimized culture (OC) medium contained the following components (g/L): sucrose, 100; (NH₄)₂SO₄, 1.5; K₂HPO₄, 5.0; NaCl, 1.0; and MgSO₄·7H₂O, 0.2, the initial pH was 6.5 for both cultures.

Culture conditions

The seed culture was prepared by inoculating cells grown on a PDA slant into 250 mL conical flasks containing 50 mL of sterilized seed culture medium and subsequently incubated at 28 °C for 48 h with shaking at 230 rpm. Then, the seed culture was inoculated into the fermentation medium, 5.0% (v/v). Batch fermentation of pullulan was conducted in a bioreactor (4×5 L, Shanghai Baoxing Bio-engineering Equipment Co., Ltd.).

Analytical methods

Determination of biomass, pullulan and residual sugar

A fermentation broth of 30 mL was centrifuged at 10,000×g for 6 min to remove the cells. Dry cell weight (DCW) was determined by drying cells at 80 °C to a constant weight. Pullulan was precipitated from the supernatant by adding two volumes of ethanol and maintaining the supernatant at 4 °C for 12 h. The precipitate was centrifuged at 8000×g for 6 min at 4 °C and dried at 80 °C overnight to a constant weight (Wang et al. 2013). The total concentration of reducing sugar was determined by the dinitrosalicylic acid (DNS) method (Miller 1959).

Characterization

Fourier transform infrared (FTIR) spectroscopy data were recorded using an attenuated total reflectance (ATR) sampling accessory (Smart iTR) equipped with a single-bounce diamond crystal on a Thermo Nicolet IS 50 spectrometer (Thermo Fisher Inc.) (Zou et al. 2013).

Molecular weight determination

Gel permeation chromatography (GPC) was conducted on a Waters Chromatography, Inc. system equipped with an isocratic pump model 1515, a differential refractometer model 2414 and a two-column set of Ultrahydrogel™ 6×40 mm Guard Column and Ultrahydrogel™ Linear 7.8×300 mm column.

Pullulan analytical standard set for GPC (Sigma, 96351-1KT; 05287-25MG; 04661-25MG) was used to make a calibration curve. 0.1 M NaNO₃ was employed as the mobile phase and the flow rate was 0.5 mL/min. 1 mg/mL of the sample concentration dissolved in eluent was used. The injection volume was 20 µL. All the data processing was carried out by Waters Breeze software (Sugumaran et al. 2013).

Enzyme activity assays of GTF

GTF activity was determined according to the method described by Duan et al. (2008). A suitably diluted cell-free extract (0.2 mL) was mixed with 0.2 mL of p-nitrophenyl-α-D-glucopyranoside (10 mmol/L) in sodium acetate buffer (0.1 mol/L; pH 4.0). The mixture was incubated at 40 °C for 5 min, and the reaction was terminated by adding 3 mL of glycine-NaOH buffer (0.4 mol/L; pH 10.5). The GTF activity was assayed based on the rate of increase in the

absorbance at 410 nm. One unit of GTF activity was defined as the release of 1 μ mol p-nitrophenol per minute at 40 °C.

Transcriptome analysis

The fermentation broth (30 mL) was centrifuged at $10,000\times g$ for 6 min to obtain the cells; then, the cells were washed twice with 50 mM Tris–HCl (pH 7.8) at 4 °C and stored at – 80 °C until RNA extraction. Total RNA was extracted using TRIzol reagent (Invitrogen). The sequencing library was built by PCR amplification and sequenced using the HiSeq™ 2500 platform (Illumina). All transcriptome data analysis was performed by the Biomarker Technologies Company.

Gene expression assays by real-time quantitative PCR

A. pullulans CGMCC No.11602 was grown in the IC and the OC media at 28 °C for 5 days, respectively. The cultures were centrifuged at $8000\times g$ and 4 °C for 10 min and the pellets obtained were used as the samples for total RNA isolation. The total RNA was purified by using a RNA prepure Tissue Kit (TIANGEN, China). The reverse transcription was conducted using a PrimeScript RT reagent Kit (TaKaRa, Japan) according to the manufacturer's protocol. All the primers used for the fluorescent real-time PCR were designed according to the corresponding gene sequences of *A. pullulans* (Liu et al. 2017; Ju et al. 2015) (Supplementary information file 1). The relative transcriptional levels of different genes were calculated using the formula $RATE = 2^{-\Delta\Delta C_t}$. The sample data obtained from the real-time PCR analysis were subjected to a one-way analysis of variance (ANOVA). *P* values were calculated by a Student's *t* test ($n=3$). *P* values less than 0.05 were considered statistically significant. A statistical analysis was performed using an SPSS 11.5 for Windows (SPSS Inc., Chicago, USA).

Results and discussion

Batch pullulan production in the fermenter

To characterize pullulan productions during the fermentation process in the IC and the OC media, samples were collected every 12 h for 4 days and analyzed for biomass, pullulan, residual sugar concentration and pullulan molecular weight distributions. Figure 1 showed significant difference in the biomass, pullulan formation and substrate consumption between the IC and the OC media. In the IC medium, the biomass, pullulan and residual sugar concentration could reach, respectively, 13.45 g/L, 64.12 g/L and 0.45 g/L, while they were only 10.34 g/L, 40.12 g/L and 28.64 g/L, respectively, in the OC medium. These results indicated the

Fig. 1 Pullulan fermentation kinetics under two different culture conditions (biomass unfilled square, pullulan unfilled triangle, residual sugar unfilled inverted triangle). The OC medium (dark gray) and the IC medium (light gray)

Fig. 2 FTIR spectra of pullulan produced in the IC and the OC media

biomass production, pullulan formation and the substrate consumption efficiency were higher in the IC medium, in which yeast extraction was used. It was also proved that the sole nitrogen source was not superior to the complex nitrogen source for pullulan fermentation based on pullulan yield (Wang et al. 2015b; Reed-Hamer and West 1994). It was believed that complex nitrogen source could provide better nutrition for cell growth and pullulan production. But no data on pullulan molecular weight were found in their reports (Wang et al. 2015a; Reed-Hamer and West 1994).

Characterization

The FTIR spectra of the two samples were similar to the pullulan standard (Sigma-Aldrich Inc.), which indicated that they were both primarily composed of pullulan (Fig. 2). Strong absorption at around 3423/cm was caused by the

–OH stretching vibration of polysaccharides. Absorption at 850/cm was characterized by the α -D-glucopyranoside units. Glucose units have been demonstrated to be linked by α -(1 $\rightarrow$ 4) and α -(1 $\rightarrow$ 6) by absorption at 755 and 915/cm, respectively. The characteristic signals obtained at 2932, 1640, and 1020/cm were attributed to C–H, O–C–O and C–O–C stretching (Chen et al. 2014). Hence, it showed that the presence of complex nitrogen source did not affect the structure of pullulan.

The distribution of pullulan molecular weight

Pullulan molecular weight was assayed every 12 h during fermentation. Figure 3 shows that the average molecular weight of pullulan increased firstly and then decreased in both the IC and the OC media. Compared with the IC medium, pullulan degradation rate was much slower in the OC medium. The pullulan molecular weight (M_w) reached 288,403 Da and 3,715,352 Da at the end of fermentation in the IC and the OC media, respectively.

In literature, it was believed that decrease in pullulan molecular weight in the later stage of fermentation was owing to pullulanase and amylase in the fermentation broth. Manitchotpisit et al. (2011) found that α -amylase attacked the minor maltotetraose subunits of pullulan and caused a reduction in pullulan molecular weight. Liu et al. (2018) found that α -amylase, glucoamylase and isopullulanase could determine the M_w of the produced pullulan. It was reported that the molecular weight of pullulan dramatically decreased after 3 days growth because of α -amylase with apparent activity in cultures (Prasongsuk et al. 2007). However, few scholars have studied the effect of pullulan synthetases on the molecular weight of pullulan. In fact, there is still not any clear regulatory mechanism related to

the decrease in pullulan molecular weight (Manitchotpisit et al. 2011).

Transcriptome analysis

To reveal the molecular weight regulation mechanism of pullulan in the IC and the OC media at the late stage of the fermentation process, the cells from 76 h fermentation, when the molecular weight of pullulan consistently decreased for both cultures (Fig. 3), were collected and analyzed by comparative transcriptome based on high-throughput sequencing. Totally, 15,525,942 and 13,704,915 raw reads were, respectively, obtained from the cells grown in the IC and the OC media. A total of 871 differentially expressed genes (DEGs) were screened from the cells in the IC and the OC media, of which 518 DEGs were up-regulated and 353 DEGs were down-regulated in the OC medium compared with the cells in the IC medium (Supplementary information file 1 and Supplementary information file 2).

The results from Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of DEGs showed that “N-glycan biosynthesis” and “other types of O-glycan biosynthesis” were the most highly represented differential metabolic pathways (DMPs) (Fig. 4). Specifically, the genes involved in these two DMPs consistently pointed to *GTF* genes, all of which were up-regulated in the OC medium compared with the IC medium (Supplementary information file 1 and Supplementary information file 2). *GTF* genes had been believed to be responsible for regulation of pullulan yield for a long time. It was also found that high glucosyltransferase activity could promote pullulan production by *A. pullulans* Y68 (Duan et al. 2008). The glucosyltransferase genes could affect the yield of pullulan and a much higher concentration of pullulan was obtained by overexpression of the glucosyltransferase gene (Chen et al. 2017). In our experiment, it was suggested that *GTF* genes may be involved in the regulation of the molecular weight of pullulan through the transcriptome analysis. To further elucidate the regulation of *GTF* genes on the molecular weight of pullulan, the activity and transcription level of *GTF* were investigated during the two fermentation processes.

The activities of GTF

The activities of GTF in different culture phases were assayed. As shown in Fig. 5, the maximum GTF enzyme activity of 6.2 U/mg was obtained at 48 h fermentation in the OC medium, which corresponded to the maximum pullulan molecular weight of 8,192,215 Da. A similar situation occurred for the culture in the IC medium where the maximum GTF enzyme activity of 5.8 U/mg presented at 48 h fermentation and the pullulan molecular weight of 1,464,103 Da was obtained. Ultimately, the GTF activities

Fig. 3 The distributions of pullulan molecular weight under two different cultures

Fig. 4 KEGG pathway enrichment analysis of DEGs between the IC and the OC media

Fig. 5 The activities of GTF in the IC and the OC media. Data are given as means $\pm$ SD, $n = 3$

were 4.74 U/mg protein and 3.4 U/mg protein, respectively, at the end of fermentation in the OC and the IC media. During both fermentation processes, the GTF enzyme activity exhibited increase first and then decrease, which was highly consistent with the variation trend of pullulan molecular weight. Moreover, the activities of GTF were remarkably higher, which were coincident with the slower decrease in the molecular weight of pullulan in the OC medium than those in the IC medium during the late stage of fermentation. The results indicated that high activities of GTF slowed

down the degradation rate of pullulan molecular weight, which may be the main reason for higher molecular weight of pullulan being produced in the OC medium.

Transcription levels of *GTF*

qRT-PCR has become the gold standard for accurate, sensitive, and rapid quantification of gene expression. *GTF* and *ACTIN* have a single peak dissolution curve in all experiment (Supplementary information file 1). It was demonstrated that there was nonspecific amplification in the experiment. All samples' transcriptional levels were analyzed in terms of the first sample in the IC medium (sample from fermentation of 24 h). In short, the RQ of the first sample in the OC medium was 1.

As shown in Fig. 6, the Ln RQ of *GTF* firstly increased to 1.66 at 48 h fermentation and then decreased to -0.16 at the end of fermentation in the IC medium, while the lowest Ln RQ of *GTF* was 0.3085 at 48 h fermentation and then continuously increased to 0.9695 at the end of fermentation in the OC medium. On the whole, the RQ values of *GTF* transcription were remarkably higher in the OC medium than those in the IC medium during the late stage of fermentation, which was in accordance with the results on GTF activity and pullulan molecular weight. However, it was unexpected that the transcription level of *GTF* continuously increased in the OC medium, which was diametrically opposite to that in the IC medium, at the late stage of fermentation. In addition, it looks like there

Fig. 6 The relative transcriptional levels of *GTF* in the IC and the OC media. Data are given as means $\pm$ SD, $n = 3$

was a turning point in the trends of the pullulan molecular weight and enzyme activity at about 48 h fermentation, where both the pullulan molecular weight and the activities of *GTF* began to decrease. It was assumed that the higher expression levels of *GTF* contributed to the higher *GTF* activities at the late stage of fermentation in the OC medium, and thus slowed down the degradation rate of pullulan.

Conclusions

Pullulan production by *A. pullulans* CGMCC No.11602 using the IC and the OC media was conducted, respectively. The differences in the fermentation processes, product characteristics, pullulan molecular weights, transcriptomics, *GTF* activities and *GTF* transcriptional levels from *A. pullulans* cells were described between the two cultures. Pullulan with significantly different molecular weight distributions but similar structural characteristics was obtained under the two cultures. In this study, we originally found that *GTF* genes were closely related to the molecular weight of pullulan, especially at the later fermentation stage. Thus, enhanced transcription and expression of *GTF* may promote high molecular weight pullulan synthesis, which provides a new idea for the production of high molecular weight pullulan.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interests regarding the publication of this paper.

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