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Pullulan production from synthetic medium by a new mutant of *Aureobasidium pullulans*

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ABSTRACT

Pullulan with different molecular-weight could be applied in various fields. A UV-induced mutagenesis *Aureobasidium pullulans* UVMU6-1 was obtained from the strain *A. pullulans* CGMCC3.933 for the production of low-molecular-weight pullulan. First, the obtained polysaccharide from *A. pullulans* UVMU6-1 was purified and identified to be pullulan with thin-layer chromatography, Fourier transform infrared, and nuclear magnetic resonance. Then, culture medium and conditions for this strain were optimized by flask fermentation. Based on the optimized medium and culture conditions (pH 4, addition of 4 g/L Tween 80 for 96 hr of cultivation), continuously fermentation was performed. The highest pullulan production and dry biomass was 109 and 125 g/L after fermentation for 114 hr, respectively. The average productivity was about 1 g/L/hr, which was intensively higher than the previous reported. This study would lay foundations for the industrial production of pullulan.

KEYWORDS

Aureobasidium pullulans; fermentation conditions; FTIR; NMR; pullulan; TLC

Introduction

Pullulan (CAS: 9057-02-7), mainly synthesized by *Aureobasidium pullulans*, is an extracellular water-soluble polysaccharide. It is composed of the repeating maltotriose units by α-(1 → 6) glucosidic linkages.[1–3] The degree of polymerization of this polymer is 100–5,000, and its molecular weight is about 1.5×10^4 to 1.2×10^7 Da. When the average molecular weight is below 2×10^5, it was usually called low-molecular-weight pullulan. Based on this special structure, pullulan endowed the polymer with distinctive physical characteristics, such as adhesive properties and the capacity to form fibers, compression moldings, and oxygen-impermeable films. Pullulan possesses numerous potential applications in cosmetics, diet food, pharmaceutical industry, adhesive, etc.[4–6] Besides, low-molecular-weight pullulan was mainly applied in cosmetics and diet food.

In spite of above advantages, the applications of pullulan were actually restricted in virtue of its production cost. Moreover, improving the pullulan titer in the fermentation broth is one of the efficient methods of production cost reduction. Therefore, many efforts were attracted in improving the pullulan titer in the fermentation process, including medium and culture condition optimization, screening of new strains, metabolic engineering of the strains, etc. Carbon sources including sweet potato, jaggery, and the hydrolysis of potato starch waste had been investigated with the pullulan production of 29.43, 51.9, and 58 g L^{-1} of culture medium, respectively.[7,8] Further research indicated that the pH of the fermentation culture medium and other factors could influence the production of pullulan efficiently.[9–11] Moreover, metabolic engineering and continuous fermentation were used to improve the production.[12–16] Much progress has been made on the production of pullulan. However, high-molecular-weight pullulan could result in high viscosity of fermentation broth at a lower titer, which is a serious hindrance to the increment of pullulan titer in the fermentation process. To avoid this problem, strains which could produce low-molecular-weight pullulan caused more attention.

In this study, a low-molecular-weight pullulan-producing strain was obtained using UV-induced mutagenesis of the strain *A. pullulans* CGMCC3.933 and named as *A. pullulans* UVMU6-1. Then the product was separated and purified. Subsequently, Fourier transform infrared (FTIR) spectroscopy, nuclear magnetic resonance (NMR), thin-layer chromatography (TLC), and gel permeation chromatography were used to verify the production from this strain. At last, the culture medium and conditions for this strain were optimized to lay foundations for industrial production.

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Materials and methods

Strains and culture conditions

The strains used in this work included *A. pullulans* CGMCC3.933 which was bought from China General Microbiological Culture Collection Center (CGMCC) and stored in the laboratory, and its UV-induced mutant, *A. pullulans* UVMU6-1. These strains were maintained at 4°C in solid Yeast extract peptone dextrose medium (YPD) medium and retrieved every month.[17,18] The YPD media contains 20 g peptone, 10 g yeast extract, and 20 g glucose per liter of culture medium. For the solid culture media, 15 g agar per liter of culture medium was added. For long-term storage, stock cultures were maintained at −80°C in a 20% glycerol solution.

UV mutagenesis

Aureobasidium pullulans CGMCC3.933 strain was cultured in a 3/15 mL of YPD medium shaken at 30°C and 180 rpm for 48 hr. Then the culture was diluted to a suitable concentration. The dilution was exposed to UV light at a distance 30–60 cm for 5 min. Then, the mutational mixtures (100 μL) were spread on the YPD solid plates, which were then cultured at 30°C for 48 hr. The mutants having a larger-colony-diameter were selected for further experiments.[18]

Precultures of the production of pullulan

The seed solutions were started by inoculating the strain into the tubes with 3 mL of YPD medium and aerobically cultured by shaking at 180 rpm and 30°C for about 36–48 hr. When the dry biomass of the culture reached 7 g/L, they were used to inoculate at 50 mL of YPD media in the 250 mL Erlenmeyer flask. Then the fermentations were done aerobically at 180 rpm and 30°C for 48 hr.

Isolation of raw extracellular product

The harvested culture of *A. pullulans* UVMU6-1 was heated at 100°C in water bath for 10 min, and cooled at room temperature. They were then centrifuged at 8,000 rpm at 4°C for 10 min. The obtained supernatant was precipitated with double volumes of precooling absolute ethanol then kept at 4°C for 12 hr, which was subsequently centrifuged at 8,000 rpm for 10 min. Finally, the precipitate was dried at 60°C to a constant weight.[19]

Purification of crude pullulan

Crude pullulan was dissolved with distilled water at 5% concentration. The solution was then washed with chloroform and *N*-butanol/amyl alcohol to precipitate proteins. The mixtures were centrifuged at 2,000 rpm for 15 min, and then the supernatant was dialyzed with deionized water for 48 hr to remove small molecules. Subsequently, pullulan was reprecipitated by adding two volumes of precooling absolute ethanol. Finally the mixtures were centrifuged at 10,000 rpm for 10 min to get the purified pullulan.[3]

Molecular weight analysis of exopolysaccharides with gel permeation chromatography

Molecular weight (M_w) of the purified exopolysaccharides (EPS) was determined by gel permeation chromatography (USA Wyatt) equipped with a TSK PW_{XL} column (USA Wyatt) and a RI detector.[20] The purified EPS (0.05 g) was dissolved in 10 mL deionized water, and centrifuged at 10,000 × *g* for 20 min before injection. The injection volume was 20 μL. Deionized water was used as a mobile phase at a flow rate of 1.5 mL/min.

Analytical methods

Verification of pullulan structure by pullulanase digestion

Suitable amount of pullulanase was added to 10 mg of purified pullulan and then hydrolyzed for 6–8 hr at room temperature. The hydrolysis mixture was spotted with a capillary pipette 1 cm apart at an origin 1.3 cm from the bottom edge on a G-60 silica gel show thin layer with maltotriose and glucose as the standard control. After the chromatography on a silica gel plate, the plate was sprayed with the appropriate agent, and heated at 105°C for about 2 min for visualization of the spots.[21]

FTIR analysis of pullulan

The IR spectroscopy was applied for further characterization of the purified pullulan.[22] FTIR samplings were performed on a Bruker (Germany) FTIR Vector 22 spectrophotometer over a range of 200–4,000 cm after blending 0.1 mg pullulan samples mixed with 200 mg KBr. The control was performed at the same conditions with the pullulan purchased from Sigma (USA).[17]

Structural analysis of pullulan by NMR spectroscopy

The NMR data were recorded on a Varian Unity 500 spectrometer (Varian NMR Systems, Palo Alto, CA, USA) operating at 500 MHz for ^1H and using 5 mm triple-resonance pulsed-field gradient probes. The samples (64 mg) were mixed with 500 μL deuterium oxides, and they were exchanged three times with D_2O, filtered, and then placed in NMR tubes (Wilmad NMR tubes, 5 mm, ultra-imperial grade, from Aldrich Chemical Company, Milwaukee, WI, USA). All the measurements were performed at 50°C and the chemical shifts were referenced to acetone ($^1H = 2.225$ ppm and $^{13}C = 31.55$ ppm).[23]

The 1D-^1H spectra of sample was acquired with a spectral width of 8,000 Hz, pulse width of 9 μs, 4 s acquisition time, and 64 scans. For the ^{13}C of NMR experiments, a spectral width of 3,000 Hz was collected. The data were zero-filled to 4,096 × 512, and a 90°C shifted sine-bell weighting function was used in both dimensions prior to Fourier transformations.

Optimization of culture conditions for pullulan production

Carbon sources, pH, and influence factors were investigated in this study to optimize the fermentation conditions. The initial fermentation medium was YPD medium which contained 10 g yeast extract, 20 g peptone, and 20 g glucose per liter of culture medium. These fermentations were performed in 50 mL of the YPD medium using 250 mL conical flasks, shaken at 30°C and

180 rpm for 72 hr. The dry biomass and the crude pullulan were subsequently determined. All the experiments were performed in triplicate.

Effects of different carbon sources on pullulan fermentation

The above seed culture was used to inoculate 50 mL of the YPD medium at 5% (without carbon source) in 250 mL conical flasks. Different carbon sources including glucose, fructose, maltose, and sucrose were then added at concentration of 4% in the culture. They were cultured at the above-mentioned conditions.

Effects of pH on pullulan production

The influence of different pH on the pullulan production was evaluated in this study. The media (addition of glucose as the carbon sources) were adjusted to different pH (3, 4, 5, 6) using either 1 M HCl or 1 M $NH_3 \cdot H_2O$ at the beginning of fermentation using YPD media in 250 mL conical flasks. They were incubated at the constant pH and 30°C on a rotary shaker for 72 hr.

Effects of influence factors on pullulan production

Influence factors play an important role in the production of pullulan. In this study, different concentrations of Mg^{2+} (0.1, 0.2, and 0.3 g/L), Tween 80 (2, 4, and 6 g/L) and vinyl ether (2, 4, and 6 g/L) were investigated. Preculture was used to incubate the culture and suitable concentration of influence factors were added to the cultures (pH 4 and glucose as the carbon source). They were then cultured and determined as mentioned above.

Fed-batch fermentation

Fed-batch fermentation was performed in a 5 L fermentor (BIOSTAT B plus MO5 L, Sartorius, Germany) using the optimized fermentation medium containing 40 g glucose, 10 g yeast extract, 20 g peptone, 5 g K_2HPO_4, 1 g NaCl, 0.6 g $MgSO_4 \cdot 7H_2O$, 0.6 g $(NH_4)_2SO_4$ per liter, and pH 4 (addition of 4 g/L Tween 80). The fermentor with 2 L of the above described production medium was sterilized at 121°C for 20 min. Meanwhile, glucose was autoclaved separately. *A. pullulans* UVMU6-1 was inoculated into 3 mL of YPD medium, cultured overnight and 100 mL of fresh YPD medium was then inoculated with above overnight cultures which were used to inoculate a production medium in the fermentor. The temperature and pH were maintained at 30°C and 4, respectively. The stirring speed was set at 400 rpm, the aeration rate was 1.5 L/min, and the oxygen pressure was set at 20%. On the course of fermentation, 70% (w/v) glucose was fed at a proper rate. Meanwhile, the yeast growth was determined by measuring the cells dry weight after a saline solution washing. The titer of crude pullulan was measured as mentioned above.[19]

Results and discussion

Molecular weight of pullulan produced by the mutant strain

The average molecular weight of pullulan was about 1.5×10^4 to 1.2×10^7 Da based on the fermentation condition and produced strains.[16] In this study, *A. pullulans* UVMU6-1 was chosen for further experiments considering its high production and small-molecular weight of product. The M_w of the purified polysaccharide obtained from the YPD fermentation broths was 6.699×10^4 Da. The polysaccharide was produced by a mutant strain. Therefore, verification of it to be pullulan was needed for further experiment.

Characterizations of pullulan produced by A. pullulans UVMU6-1

Subsequently, the purified polysaccharide was hydrolyzed with pullulanase and then analyzed with TLC to identify the product. Furthermore, FTIR analysis and NMR spectroscopy analysis were also performed to verify the sample.

Verification of products with enzymolysis and TLC

Pullulanase could selectively hydrolyze α-(1 → 6) glucoside bonds of pullulan to form maltotriose.[24] Therefore, the purified sample and the standard pullulan were hydrolyzed with pullulanase and then analyzed with TLC. Based on the results of TLC (Figure 1), the sample was composed by maltotriose joined by α-(1 → 6) glucoside linkages. These results indicated that pullulan was the major component of the samples.[25]

FTIR analysis

To further verify the sample, both the standard and the purified sample from the broth culture were subjected to FTIR, and their spectra were shown in Figure 2. Intense absorption at 3,400/cm was caused by the –OH stretching vibration of polysaccharides. Meanwhile, absorption at 840/cm is characterized by the α-D-glucopyranoside units.[26] Glucose units have been shown to be linked by α-(1 → 4) and α-(1 → 6) by absorption at 755 and 915/cm, respectively. The characteristic obtained signals at 2,932, 1,640, and 1,020/cm were attributed to C–H, O–C–O, and C–O–C stretching, respectively.[27] The purified and standard sample appeared the same characteristic

Figure 1. Thin layer chromatography of the products hydrolyzed with pullulanase. Lane 1: the hydrolyzed standard pullulan (1 g/L). Lane 2: the standard pullulan (1 g/L). Lane 3: maltotriose (10 mg/L). Lane 4: the hydrolyzed sample (1 g/L). Lane 5: the sample (1 g/L).

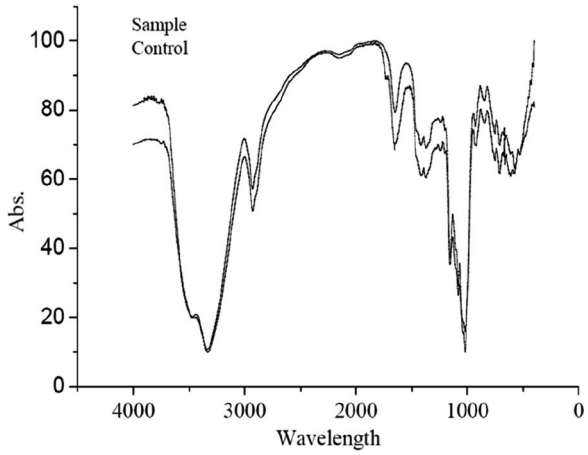

Figure 2. FTIR spectra of pullulan produced with glucose as the carbon source. Glucose units have been shown to be linked by α-(1 → 4) and α-(1 → 6) by absorption at 755 and 915/cm, respectively. The characteristic signals obtained at 2,932, 1,640, and 1,020/cm were attributed to C–H, O–C–O, and C–O–C stretching. This result indicated that the purified sample and standard sample appeared the same characteristic peaks of pullulan. *Note*: FTIR, Fourier transform infrared.

peaks of pullulan, which indicated that the purified sample was pullulan.

NMR spectroscopy analysis

Subsequently, the purified sample was further identified with ^{1}H NMR and ^{13}C NMR, and then the spectrum was compared with that of the standard. The 1D ^{1}H and 1D ^{13}C spectra of pullulan from UVMU6-1 are shown in Figure 3a and 3b. The proton peak displacement of the purified sample appears between $W_{3.3}$ and $W_{5.4}$ (^{1}H of NMR), and between $W_{20.0}$ and $W_{220.0}$ (^{13}C of NMR). The spectra of the purified sample were the same as those of the standard, which indicates that they were both pullulan. From these characteristic peaks of the ^{1}H and ^{13}C of NMR spectra and the results of TLC and FTIR analysis, we could conclude that the purified sample was pullulan.

Optimization of the fermentation media and culture conditions

Fermentation medium and culture conditions play a vital role in the concentration and yield of the end product. Moreover, they also provide data for fed-batch fermentation. In this study, one-factor-at-a-time method was applied to optimize medium components and process conditions. The most important factors including carbon source, pH, influence factors was optimized to improve pullulan production.

Effects of carbon sources on pullulan production

Several studies have shown that different carbon sources have profound influences on EPS yield by bacteria.[28,29] Therefore, the influence of different carbon sources on the dry biomass of *A. pullulans* UVMU6-1 and the pullulan production were investigated, including glucose, fructose, maltose, and sucrose. The results were shown in Figure 4. The glucose (4%) permitted highest pullulan production as well as dry biomass of UVMU6-1 than others. When glucose was used as sole carbon source, the highest concentration of pullulan was 19.8 g/L in

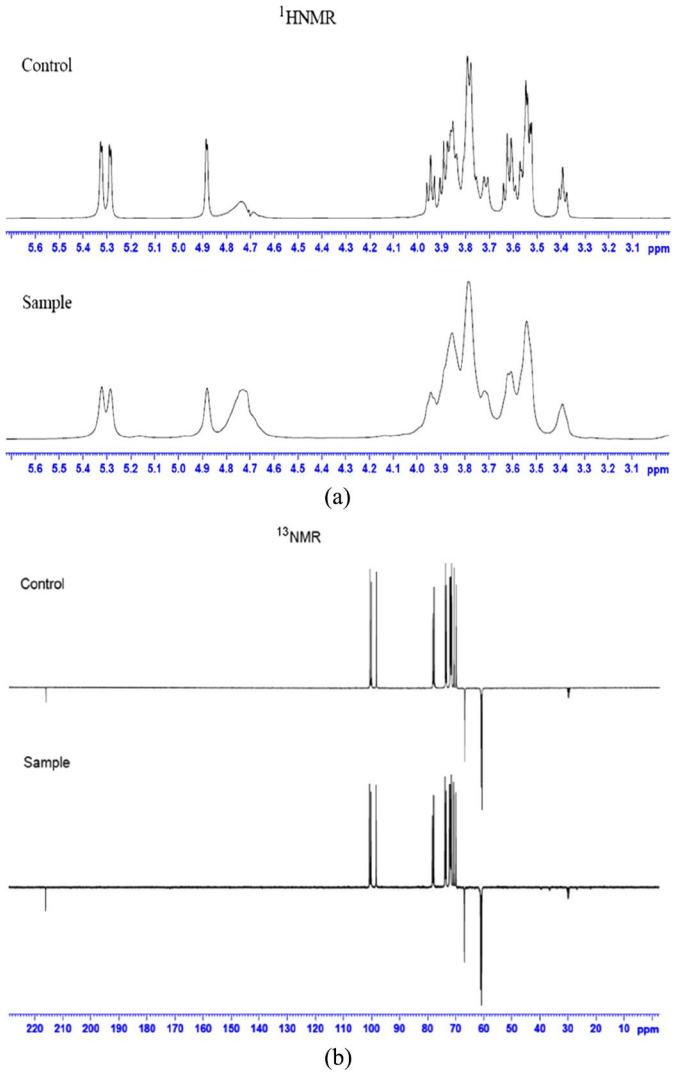

Figure 3. NMR spectra of pullulan isolated from fermentation broths and the pullulan (Sigma, USA). (a) The 1D ^{1}H spectra of pullulan from UVMU6-1 (control and sample). (b) The 1D ^{13}C spectra of pullulan from UVMU6-1 (control and sample). The displacement of proton peak of the purified sample appears between $W_{3.3}$ and $W_{5.4}$ (^{1}H of NMR), and between $W_{20.0}$ and $W_{220.0}$ (^{13}C of NMR). The spectra of the purified sample were the same as those of the standard.

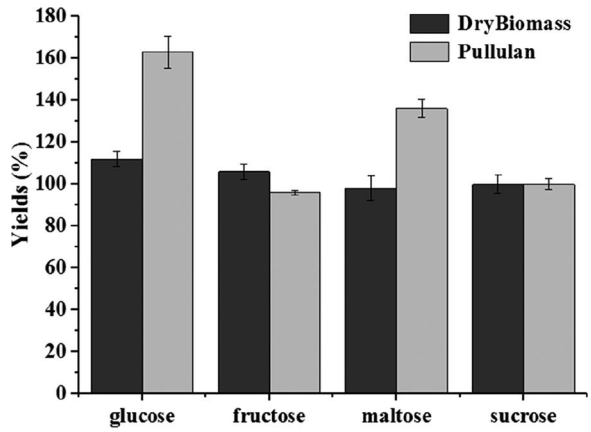

Figure 4. Effects of carbon sources (glucose, fructose, maltose, and sucrose) on pullulan production using conical flasks. The carbon source was optimized to be glucose. To make the results more visual, the sample with the lowest pullulan production was defined as 100%.

the culture medium. Meanwhile, when fructose and sucrose were used as carbon sources, the *A. pullulans* UVMU6-1 dry biomass was higher than that with maltose, yet a lower production of pullulan were obtained from fructose and sucrose enriched media. Therefore, glucose was the suitable carbon source not only for the dry biomass but also for the production of pullulan with *A. pullulans* UVMU6-1.

Influence of pH on pullulan production

The pH of fermentation broth affected the morphology of the organism, which may further influence pullulan production.[30] In this study, the effect of pH on the pullulan production and the dry biomass of *A. pullulans* UVMU6-1 were investigated and the results were presented in the Figure 5. Unlike the reports by Lacroixt et al.,[2] dry biomass and pullulan production influenced by pH were moderate in this study (Figure 5). At the pH of 3, the dry biomass and the pullulan production were lower with dry biomass and pullulan concentration of 28 and 6 g/L, respectively, after 72 hr. The highest yield was obtained at the pH of 4 with a maximum dry biomass and production of 39 and 23.2 g/L, respectively. However, the yields of pullulan decreased when increased the pH.

In several reports the influence of different pH ranges varying from 3 to 6 of the fermentation medium on production was performed.[31,32] Previous works reported that the optimal pH values of fermentation media for pullulan production were ranged from 4.5 to 8.5. The variations in optimal pH might be caused by variations in type of strains, composition of fermentation medium. and growth conditions.[8] However, the optimal pH in this study was 4 when glucose was used as a sole carbon source for the production of pullulan through the strain *A. pullulans* UVMU6-1. Therefore, all subsequent experiments were performed at pH 4.

Effects of influence factors on the production of pullulan

Mg^{2+}, Tween 80, and vinyl ether were investigated to improve the yields of pullulan in this study.

Varying concentrations of Mg^{2+} were added into the medium. As can be seen in Figure 6a, the influences of Mg^{2+} on the production of pullulan as well as the dry biomass were not obvious. It is well known that Mg^{2+} is an important coenzyme and the essential element of numerous enzymes. Moreover, the different concentrations of metal ions also could affect the activity of enzymes.[33] However, Mg^{2+} was not the key factors on the pullulan production which would reduce the cost of the medium.

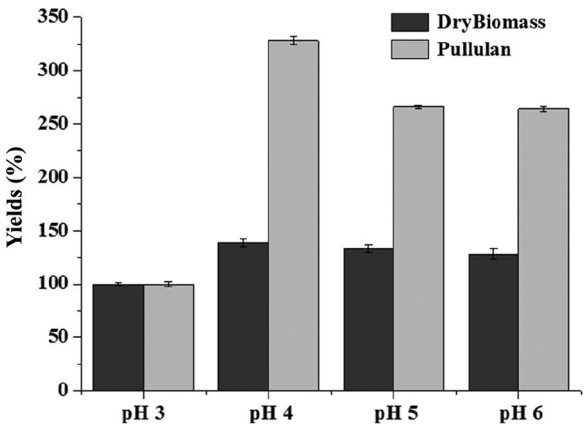

Figure 5. Influence of pH on pullulan production. The media were adjusted to different pH (3, 4, 5, 6) using either 1 M HCl or 1 M $NH_3 \cdot H_2O$. The optimized pH of the media was 4. To make the results more visual, the sample as pH 3 was defined as 100%.

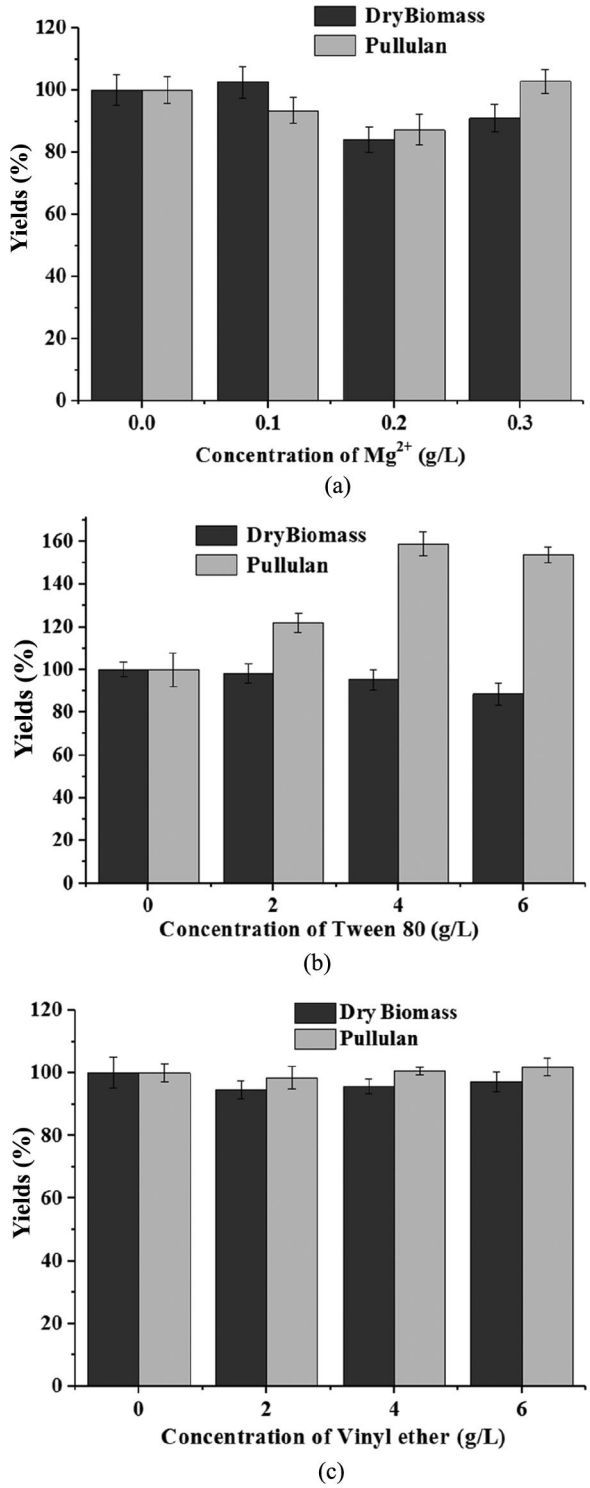

Figure 6. Effects of influence factors on the production of pullulan. (a) The influences of Mg^{2+}(0.1, 0.2, and 0.3 g/L); (b) the influences of Tween 80 (2, 4, and 6 g/L); (c) the influences of surfactant vinyl ether (2, 4, and 6 g/L). The control one was defined as 100%. The key factor on the production of pullulan was Tween 80 and the concentration of 4 g/L.

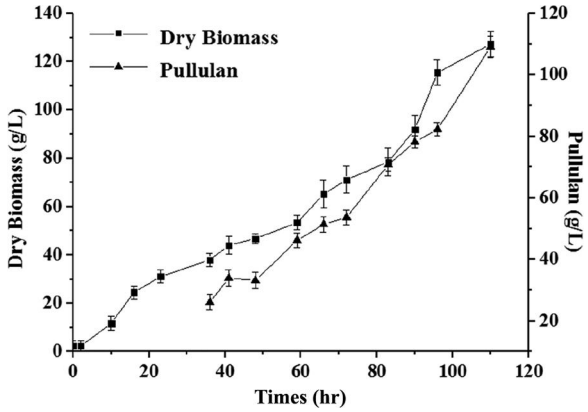

Figure 7. The dry biomass and pullulan-producing of UVMU6-1 under optimized conditions of fed-batch fermentation in a 5 L fermentor (when the fed-batch fermentation of UVMU6-1 was performed at 96 hr at the dry biomass of 92, 4 g/L Tween 80 was added to the culture including glucose. The maximal production of pullulan and dry biomass were 109 and about 125 g/L after fermentation for 114 hr, respectively, with an average productivity of about 1 g/L/hr).

Different concentrations of Tween 80 (2, 4, and 6 g/L) were added to the media to evaluate their effects on pullulan production by *A. pullulans* UVMU6-1. As can be shown in Figure 6b, the addition of Tween 80 to the media could significantly increase the production of pullulan without the improvement of biomass. Maximum pullulan production was observed at the Tween 80 concentration of 4 g/L in the medium at 180 rpm and 30°C for 48 hr. Meanwhile, the dry biomass was slightly less than the control group. Tween 80 is an excellent surfactant and could increase the permeability of the lipid bilayer membrane of the cell. Tween 80 may play a role of an enhancer of not only the pullulan transporting into the media from the cell bodies but also nutrient uptake from the media into the cell bodies.[34] Furthermore, it is well-known that some bacteria can utilize Tween 80 as carbon sources,[35–37] which would be helpful for the fermentation. Meanwhile, the effects of surfactant vinyl ether on the production of pullulan were not obvious (Figure 6c). Therefore, Tween 80 was used as additions to improve the pullulan production in the further fermentation.

Fed-batch fermentation in a 5L fermentor

The fermentation kinetics of UVMU6-1 with glucose-containing medium under the optimized conditions (pH 4, addition of 4 g/L Tween 80 after cultivation for 96 hr) was shown in Figure 7. The maximal production of pullulan and dry biomass were 109 and 125 g/L, respectively, after 114 hr of fermentation, with an average productivity of about 1 g/L/hr, which was intensively higher than that reported before.[38] As can be seen in Figure 7, after adding Tween 80 at 96 hr, the cells growth was slightly inhibited and the concentration of pullulan rose phenomenally. Tween 80 was considered responsible for pullulan synthesis just as observed previously.[39] The results suggested that the low-molecular-weight pullulan production could be significantly improved under optimized conditions, which would lay foundations for the industrial production.

Conclusion

In this study, we achieved to obtain a mutant strain, *A. pullulans* UVMU6-1, which could produce a polysaccharide with the molecular of 6.699×10^4 Da. The polysaccharide was identified to be pullulan with TLC, FTIR, and NMR. Subsequently, the medium and culture conditions for this strain were optimized. Based on the optimized conditions (pH 4, addition of 4 g/L Tween 80 after cultivation for 96 hr), continuously fermentation was performed with a maximal yield of pullulan and dry biomass 109 and 125 g/L, respectively, which was intensively higher than that reported before. The average productivity was 1 g/L/hr. The results provided a more economical strain and way to produce low-molecular-weight pullulan at industrial scale.

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