JOURNAL OF PURE AND APPLIED MICROBIOLOGY, March 2015. Vol. 9(1), p. 17-22

Influence of pH on Pullulan Biosynthesis and the Molecular Weight by *Auerobasidium pullulans*

Jianzi Wang[1], Fangyan Sun[1], Yaqiong Song[1], Jianming Wang[1], Huaxuan Hao[2], Zhenhai Li[2] and Changsheng Qiao[1, 2]

[1]Key Laboratory of Industrial Microbiology, Ministry of Education, Tianjin University of Science and Technology, Tianjin, 300457, China.
[2]Tianjin Peiyang Biotrans Co., Ltd, Tianjin, 300457, China.

(Received: 10 December 2014; accepted: 17 January 2015)

Pullulan is an extracellular water soluble polysaccharide, which has been considered as a promising biodegradable material. And its derivatives have been of interest in past few years in a broad range of industrial fields including the food, cosmetics. The effects of culture pH ranging from 2.0 to 7.0 on cell growth, pullulan biosynthesis, the activity of pullulan-degrading enzyme, and the molecular weight of pullulan were investigated. The optimal pH for biomass formation was around 3.0, whereas the value for pullulan production was around 5.0. The portion of high molecular weight pullulan declined with fermentation time. In addition, when the pullulan-degrading enzyme was detected, its activity increased until the end of the fermentation, which caused the molecular weight to decrease faster, indicating that the pullulan-degrading enzyme was correlated with the decreasing molecular weight of pullulan. A dual-stage pH process that maximizes product formation has been successfully demonstrated with a high product yield of 78.12g/L with the relatively high average molecular weight of 2.02 × 10^5Da.

Key words: Pullulan biosynthesis; Pullulan-degrading enzyme; Dual-stage; Molecular weight; pH.

Pullulan is an extracellular water soluble polysaccharide produced by the yeast-like fungus *Aureobasidium pullulans*. It is a neutral polymer of repeating glucose units with two different glycosidic bonds (±-1, 6 and ±-1, 4). Its molecular weight was ranging from 4.5 × 10^4 to 2 × 10^6 Da. Potential applications of pullulan and its derivatives have been of interest in past few years in a broad range of industrial fields including the food, cosmetics. [1]And high molecular weight pullulan seem to be more effective than those of low molecular weight.[2]

* To whom all correspondence should be addressed.
Tel: +86-22-60601606; Fax: +86-22-60602298;
E-mail addresses: qiaochangsheng@tust.edu.com

The pH of culture broth is one of the most critical environmental parameters affecting growth and biosynthesis of exopolysaccharides in submerged cultures. However, the influence of pH on the biosynthesis of exopolysaccharides and cell growth varies with different microorganisms [3-6]. In general, the optimal medium pH for cell growth is around the lower range from 2.0 to 4.0 but the optimal medium pH for exopolysaccharide formation is around the high range from 5.0 to 7.0[7-11]. And Catley [12] first illustrated the pH effect of pullulans on production. The results showed that the optimal pH for pullulan synthesis and cell mass growth is different. Other studies reported that pullulan-degrading enzyme may appear at the late stage of pullulan fermentation, which results in a decrease of pullulan production.[13,14] However, till now the relationships between pullulan-degrading enzyme activity and the molecular weight have not been studied.

Therefore, it is of our interest to investigate the influence of pHand fermentation timeon pullulan production, biomass, pullulan-degrading enzyme activity, and the molecular weight of *A. pullulans* CGMCC7055.

MATERIALS AND METHODS

Microorganism

Yeast like fungal strain *A. pullulans* CGMCC7055, preserved in China General Microbiological Culture Collection Center (Institute of Microbiology, Chinese Academy of Sciences, Beijing, 100000, China), was used. Stock cultures were maintained on potato sucrose agar at 4°Cand were subcultured for every month.

PH control and culture conditions

The effect of pH on the fungus culture was studied by batch fermentation in a 5-L bioreactor (Shanghai, China) with pH control. A range of culture pH was examined from2.0 to 7.0 in steps of 1.0 pH unit. The medium used in this study contained the following components (g/L): sucrose 150, peptone 5, K_2HPO_4 7, $MgSO_4 \cdot 7H_2O$ 0.4, NaCl 3 and $FeSO_4 \cdot 7H_2O$ 0.05. The medium pH was adjusted by adding 1 M NaOH or 1 M HCl prior to sterilization.

The inoculum of 100 mL was prepared by flask culture at 32°C and 180 rpm for 30h. The medium for inoculum preparation (seed medium) contained the following components (g/L): sucrose 100, yeast extract 3, $(NH_4)_2SO_4$ 1, K_2HPO_4 2, $MgSO_4 \cdot 7H_2O$ 0.4, NaCl 2.5 and $FeSO_4 \cdot 7H_2O$ 0.05, pH 7.0. The fermentation with 3.5 L of medium was operated at temperature 28°C, 1vvm aeration and agitation 400 rpm for 88h. A pH shift experiment was demonstrated by controlling the culture pH at 3.0 for cell growth in the first stage then at 5.0 for product formation.

Analytical methods

Biomass

To determine biomass, five milliliter of broth was centrifuged at 2000×g at 4°Cfor 20 min (Microfuge 18, Beckman Coulter Co., Fullerton, CA). Then, the pellets were washed twice with distilled water and centrifuged again to remove impurities. After the pellets were dried at 80°Covernight(>8h) , dry cells weight(DCW) was determined as biomass[10].

Extraction of pullulan

Five milliliter fermentation broth was also centrifuged at 3300×g at 4°Cfor 20 min(Microfuge 18, Beckman Coulter Co., Fullerton, CA) to remove the microorganisms. Three milliliter of supernatant was then mixed with six milliliter of 95% ethanol and gently stirred. The resulting precipitate was dissolved in equal volumes water and purified the pullulan by twice ethanol precipitation. The precipitate was dried at 80°Cuntil its weight was constant, then its dry weight was determined. [15]

Sugar analysis

The sugar concentration was measured in the cell free broth using Miller's method.[16]

Determination of molecular weight of pullulan by GPC

The molecular weight (M_W) of pullulan sample was estimated using gel permeation chromatography (Agilent 1200 series, USA) equipped with a PL gel column of 5¼m pore size (Viscotek, USA) and RI detector. 0.05M Na_2SO_4 was used as a mobile phase at a flow rate of 0.5 ml/min. The sample concentration and injection volume were 2.0 mg/mL and 10¼L. All of the sample solutions were filtered through 0.45-lm-pore-size filters (Adbentec MFS, Inc., Japan) before injection. Pullulan standards with the molecular weights ranging from 7.00×10^4 to 1.60×10^6 Da were used to construct a calibration curve.[11]

Activity of pullulan-degrading enzyme

Samples at 40h, 52h; 64h, 76h; 88h respectively were centrifuged at 2000×g, 4 °C for 30 min to produce cell-free fermentation broth for enzyme assays.

The pullulan-degrading activity was assayed by adding 1 mL of fermentation broth into a reaction mixture containing 1 mL of 1% pure pullulan standard in 50 mM sodium acetate buffer at pH5.0, 50 °Cfor 40min. Fermentation broth, which was inactivated usingboiling water for 5 min, was used as control. The reaction washalted by heating the assay mix at 100 °C for 8 min. Reducing sugar content was determined using Miller's method. The calibrationcurve used for reducing-sugar determination was generated byusing pure pullulan. The activity of pullulan-degrading enzyme was expressed as IU: defined as 1¼mol glucose equivalents liberated per min per mL of fermentation broth at 50 °C. [13,17]

RESULTS AND DISCUSSION

Effect of pH control on cell growth and pullulan formation

pH levels during the fermentation process have the potential to influence the morphology of *A. pullulans*, which in turn will also influence the cell growth and pullulan production[18]. Therefore, this work investigated the effects of various pH levels (ranging from 2.0 to 7.0 and increasing in 1.0 pH increments) on cell growth and pullulan formation during CGMCC7055-induced fermentation.

As time passed during the adjustment stage, each pH values' specific growth rate (¼) increased, but not at the same rate for each value. The results showed that for the pH value of 3.0 the adjustment stage was the shortest and that a value of 13.56 g/L was the optimal for the maximum cell density (X max). Interestingly, the X max decreased as the pH increased. The maximum specific growth rate at a pH of 3.0 was 0.19 h^{-1}, which was less than that at a pH of 2.0, while the optimal cell production rate (Q_X) (0.61gL^{-1} h^{-1}) was found to occur at the pH of 3.0, and this also decreased as the pH value increased (Table 1). From these results it was possible to determine that a pH value of 3.0 was the best for cell growth as higher pH values could inhibit the formation of cells.

While cell growth showed a distinct exponential phase and a stationary phase, pullulan biosynthesis occurred throughout the culture (Fig. 1). At pH 5.0, although cell growth is not the best, pullulan produced far ahead in all fermentation processes (Fig. 1), and the maximum pullulan concentration (P max) of pH 5.0 showed an optimal value 68.78 g/L, the biggest among the controlled pH processes. Like-wise, the optimal production formation rate (Q_p) also occurred at pH 5.0 was 2.15 gL^{-1} h^{-1}. Higher or lower culture pH than 5.0 inhibited the formation of pullulan. These results are agreed with Cheng.[15]

Effect of pH on the molecular weight of pullulan, sugar concentration and pullulan-degrading enzyme activity

To elucidate the effect of pH on the molecular weight of pullulan, the molecular weight was characterized by the weight-average, Mw.[19]

Results are all shown in Fig. 2. The effect of the pH was qualitatively determined from the changes in Mw observed when the pH values were altered. For each controlled pH value, the Mw decreased with the passage of fermentation time, and decreased more rapidly when the pullulan-degrading enzyme was detected. The activity of the pullulan-degrading enzyme was the biggest at pH7. This result hasn't been reported now. Under each controlled pH value, when the sugar concentration was higher than 10g/L, the pullulan-degrading enzyme could not be detected.

As the pH was adjusted from 2.0 to 7.0, the Mw of pullulan not only decreased monotonically from 7.58×10^5 to 4.12×10^5 at 40h, but also decreased monotonically from 4.12×10^5 to 1.14×10^5 after 88h of fermentation. When the pH was lower it was possible to obtain a higher Mw of pullulan.This observation was consistent with that of pullulan fermentation by Madi et al[20]. However, in an alternative report the results contradicted this when a high Mw portion of pullulan was obtained from fermentation at a pH of 6.5.[21] This might be due to differences in the

Table 1. Fermentation parameters of the batch experiments under various pH controlled processes in a stirred tank fermenter

Different pHcontrolled processes	μ (h^{-1})	Q_X (gL^{-1} h^{-1})	Q_P (gL^{-1} h^{-1})	X_{max} (gL^{-1})	P_{max} (gL^{-1})
Controlled at pH 2.0	0.28	0.56	1.34	12.02	38.20
Controlled at pH 3.0	0.19	0.61	2.01	13.56	51.30
Controlled at pH 4.0	0.15	0.57	2.09	11.73	62.35
Controlled at pH 5.0	0.14	0.53	2.15	11.22	68.78
Controlled at pH 6.0	0.11	0.41	1.46	10.30	56.27
Controlled at pH 7.0	0.10	0.39	1.39	8.15	53.20
Two-stage: pH 3.0'!pH 5.0[a]	0.23	0.55	2.21	16.63	78.12

[a] Culture pH was controlled at pH 3.0 within the first 40h and then shifted to pH 5.0 until the end of the fermentation.

microorganism and other environmental conditions, for instance oxygen transfer rate and shear rate.

It could be seen that the pullulan-degrading enzyme appeared in the culture medium when the sugar concentration was lower than 10g/L, while its activity increased until fermentation ended. For each controlled pH value, the rate of the Mw decrease was greater as the pullulan-degrading enzyme activity increased. There was a correlation between the pullulan-degrading enzyme activity and decreases in the Mw of the pullulan.

Fermentation time was also a factor affecting the Mw of the pullulan as shown in Fig. 2. The high molecular weight portion of the pullulan declined with fermentation time, and this was consistent with previous pullulan fermentations[20,21]. In fact, there was no higher molecular weight pullulan present at the end of the fermentation. Thus, if higher molecular weight pullulan was desired, the fermentation time should be minimized. This result also indicated that other crucial enzymes influencing the Mw of pullulan also existed.

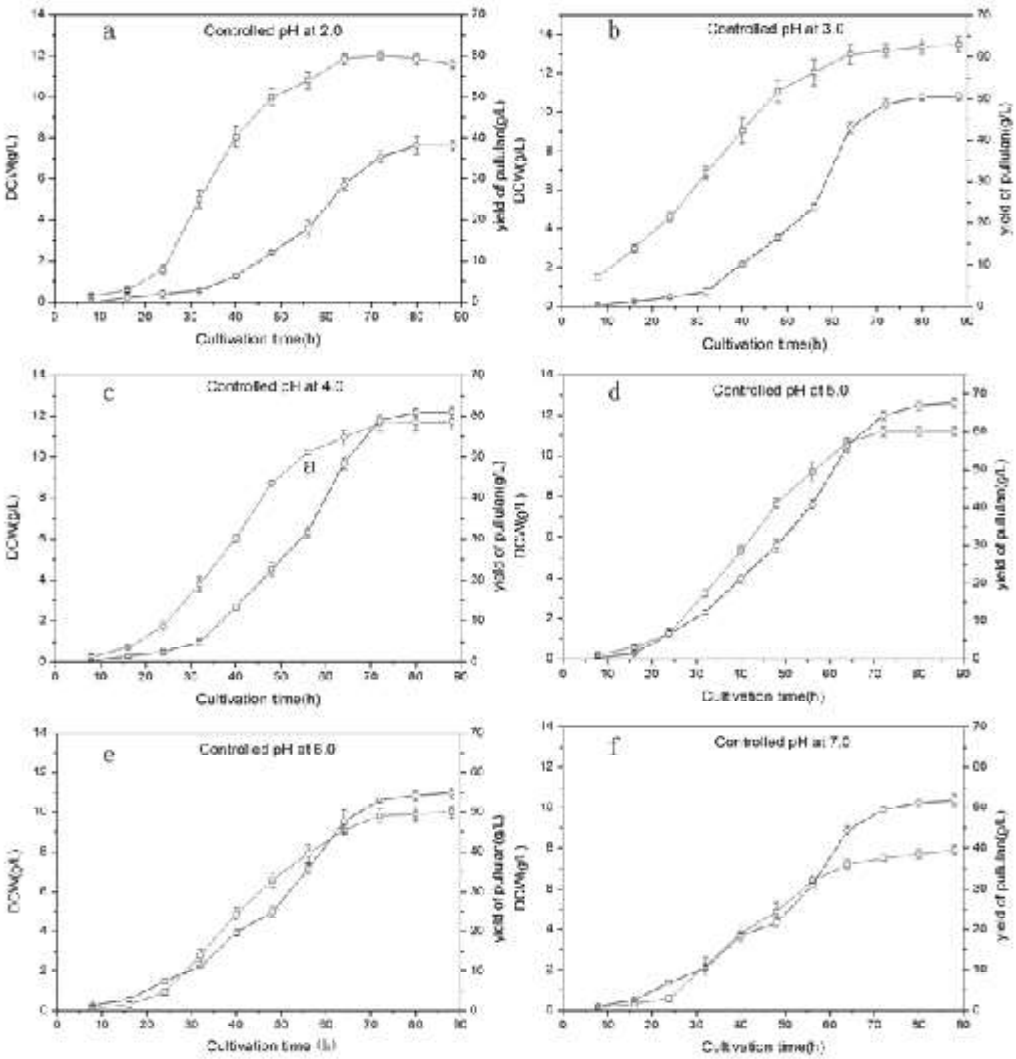

Fig.1. Time-course data of pH controlled batch fermentation in a stirred tank fermenter with set point at (a) pH 2.0; (b) pH 3.0; (c) pH 4.0;(d) pH 5.0; (e) pH 6.0; (f) pH 7.0, respectively. Yield of pullulan (Ë%); DCW (¡%)

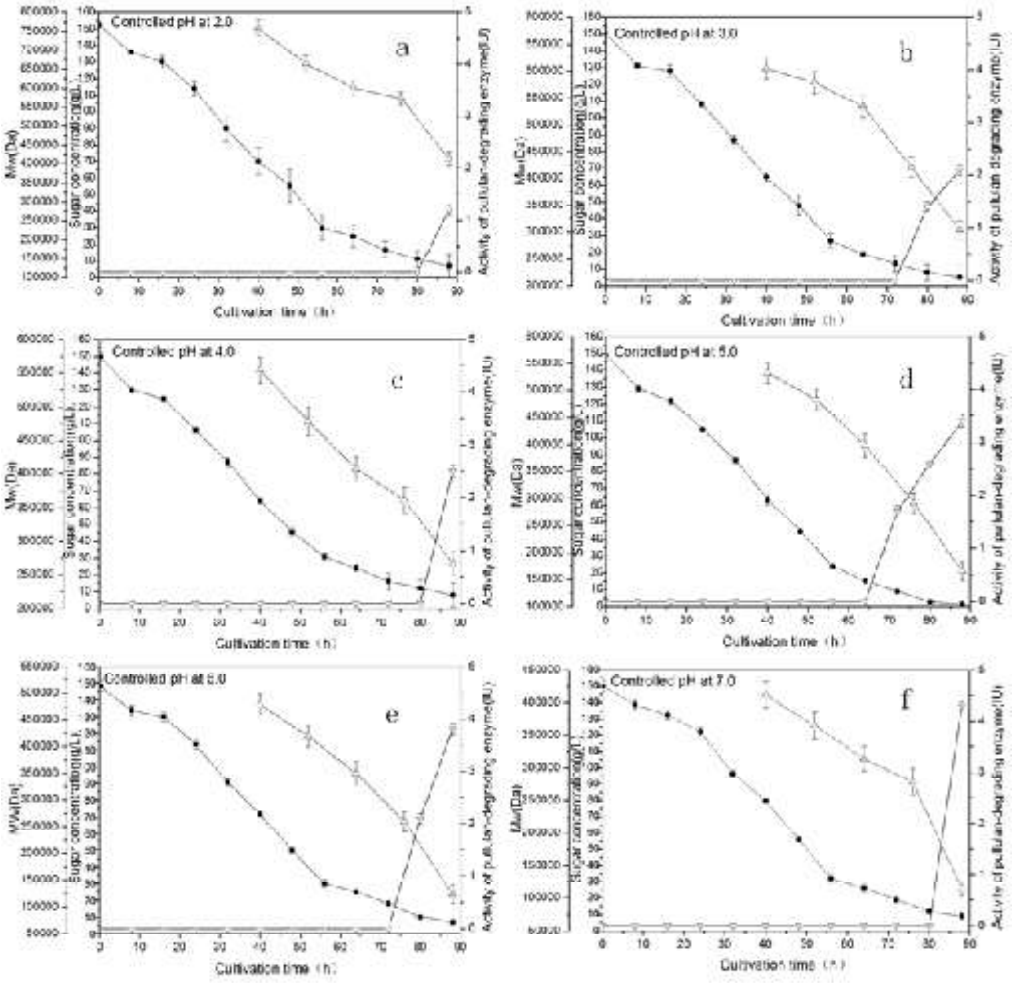

Fig. 2. Time-course data of pH controlled batch fermentation in a stirred tank fermenter with set point at (a) pH 2.0; (b) pH 3.0; (c) pH 4.0;(d) pH 5.0; (e) pH 6.0; (f) pH 7.0, respectively. Sugar concentration (Ï%); Mw (³%); Activity of pullulan-degrading enzyme(½%)

Dual-stage batch fermentation process for optimal pullulan production

The fermentation process in a stirred tank with dual-stage pH operation was performed to optimize pullulan production as demonstrated in Fig.3. In the first stage, the culture pH was controlled at a pH of 3.0 for around 40h to enable cell growth. Then in the second stage pullulan production was initiated via a shift to a pH of 5.0. The details of the fermentation parameters are listed in Table 1.

As expected, pullulan production in the dual-stage batch fermentation process was enhanced by 13.58% when compared to that of fermentation at pH 5.0, reaching 78.12g/L. The Mw of the dual-stage batch process after 88h of fermentation was 2.02×10^5Da, which was slightly greater than that of the pH controlled fermentation at a pH value of 5.0. This means that the change in the pullulan's Mw was also obtained via the dual-stage process.

CONCLUSION

In a word, the culture pH in stirred tank fermentation of *A. pullulans* has a critical influence on cell growth, pullulan formation and Mw. The optimal pH for biomass formation was around 3.0, whereas the value for pullulan production was around 5.0. High molecular weight pullulan was

obtained at lower pH values while low molecular weight pullulan was obtained at higher pH values.

The portion of high molecular weight pullulan declined with fermentation time. In addition, when the pullulan-degrading enzyme was detected, its activity increased until the end of the fermentation, which caused the Mw to decrease faster, indicating that the pullulan-degrading enzyme was correlated with the decreasing Mw of pullulan. The Mw of pullulan was also influenced by other significant enzymes as well.

A dual-stage pH process that maximizes product formation has been successfully demonstrated. This dual-stage pH fermentation process offers certain advantages including high product yields with relatively higher molecular weights.

ACKNOWLEDGEMENTS

This work was financially supported by the Project of Tianjin university of Science and Technology Support Program and the company of tianjin Peiyang Biotrans. The authors would like to thank these organizations for their kind financial support.

REFERENCES

1. Prajapati VD, Jani GK, Khanda SM. *Carbohydr Polym,* 2013; **95**: 540-9.
2. Yu X, Wang Y, Wei G, Dong Y. *Carbohydrate Polymers,* 2012; **89**: 928-934.
3. Cheng K-C, Demirci A, Catchmark JM. *Applied microbiology and biotechnology,* 2010; **86**: 853-861.
4. Fang Q-H, Zhong J-J. *Process Biochemistry,* 2002; **37**: 769-774.
5. Ono K, Yasuda N, Ueda S. *Agricultural and Biological chemistry,* 1977; **41**.
6. Wang Y, McNeil B. *Enzyme and Microbial Technology,* 1995; **17**: 124-130.
7. Cheng KC, Demirci A, Catchmark JM. *Applied microbiology and biotechnology,* 2011; **92**: 29-44.
8. Lee KY, Yoo YJ. *Biotechnology letters,* 1993; **15**: 1021-1024.
9. Li B-x, Zhang N, Peng Q, Yin T, Guan F-f, Wang G-l, Li Y. *Applied microbiology and biotechnology*, 2009; **84**: 293-300.
10. Shingel KI. *Carbohydrate research*, 2004; **339**: 447-460.
11. Pan S, Yao D, Chen J, Wu S. *Carbohydr Polym,* 2013; **92**: 629-32.
12. Catley BJ. Applied microbiology, 1971; **22**: 641-649.
13. Cheng K-C, Demirci A, Catchmark JM, Puri VM. *Journal of Food Engineering,* 2011; **103**: 115-122.
14. Zheng W, Campbell BS, McDougall BM, Seviour RJ. *Bioresource technology*, 2008; **99**: 7480-7486.
15. Cheng K-C, Demirci A, Catchmark JM. *Applied microbiology and biotechnology,* 2011; **92**: 29-44.
16. Miller GL. *Analytical chemistry,* 1959; **31**: 426-428.
17. West TP, Strohfus B. *Journal of basic microbiology*, 1996; **36**: 377-380.
18. LeDuy A, Marsan A, Coupal B. *Biotechnology and Bioengineering*, 1974; **16**: 61-76.
19. Petrov P, Shingel K, Scripko A, Tsarenkov V. *Biotekhnologiya*, 2002; **1**: 36-48.
20. Madi NS, McNeil B, Harvey LM. *Journal of chemical technology and biotechnology*, 1996; **65**: 343-350.
21. Lee J-H, Kim J-H, Zhu I-H, Zhan X-B, Lee J-W, Shin D-H, Kim S-K. *Biotechnology letters,* 2001; **23**: 817-820.