

## Effect of Two-Stage Controlled pH and Dissolved Oxygen Concentration on Pullulan Production by *Aerobasidium pullulans*

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Effect of two-stage controlled pH and dissolved oxygen concentration (DO) on pullulan production was investigated. Lower pH and higher DO controlled supported the cell growth of *Aerobasidium pullulans* CGMCC7055, while higher pH and lower DO controlled stimulated pullulan synthesis. Maximum pullulan production (80.67g/L) was obtained at initially lower pH (3.0) and higher DO (50%), subsequently higher pH (5.0) and lower DO (30%). And was 17.59% (w/w) and 16.07% (w/w) more than those achieved at a two-stage pH (initially pH 3.0 and subsequently 5.0) with uncontrolled DO, and a two-stage DO (initially 50% and subsequently 30%) with uncontrolled pH (initial pH 6.0). Results indicated that combination of the two-stage controlled pH and DO significantly supported mass production of pullulan. This new and improved pullulan production process methodology would 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.

**Key words:** Pullulan; Two-stage; pH; Dissolved oxygen concentration.

Pullulan is an extracellular water soluble polysaccharide produced by the yeast-like fungus *Aerobasidium pullulans*. It is a neutral polymer of repeating glucose units with two different glycosidic bonds ( $\alpha$ -1, 6 and  $\alpha$ -1, 4) and without branching. 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.

For pullulan production, the effect of culture pH and DO has drawn much attention. The pH of culture broth is one of the most critical

environmental parameters affecting growth and biosynthesis of exopolysaccharides in submerged cultures. However, the effect of pH on the biosynthesis of exopolysaccharides and cell growth varies with different microorganisms<sup>1-4</sup>, operational conditions and medium composition. 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<sup>5-9</sup>. And Xia *et al.*<sup>10</sup>, has indicated the optimal pH of cell growth and pullulan production is different, and lower pH of the culture medium is benefit to cell growth of *A. pullulans*, while higher pH of the culture medium supports mass pullulan production.

DO is also a critical environmental factor in the process of microbial fermentation. It plays a

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very important role in cell growth and product synthesis. Too high or too low DO level is not good to cell growth and product synthesis. Under no dissolved oxygen concentration conditions, the cell population neither grows nor produces pullulan. The accumulation of pullulan synthesized during the course of fermentation results in a highly viscous and non-Newtonian broth which in turn limits heat and mass transfer<sup>11</sup>. Consequently, DO is no longer maintained at the preset maximal level. It is not known whether such a decrease in oxygen tension has any significant effect on the synthesis of pullulan. And Rho *et al.*<sup>12</sup> firstly reported that DO was essential for pullulan synthesis and the requirement of DO concentration for cell growth was not in accordance with that of pullulan production.

However, these researches did not include the information on double control of pH and DO. There are few reports on the control strategies in stages by combining pH and DO. This work mainly aimed to study the effects of pH and DO on cell growth and the pullulan production, and developed a combined pH and DO control strategy to enhance pullulan production. The control strategy proposed here may be helpful to the production of the other exopolysaccharides.

## MATERIALS AND METHODS

### Microorganism

*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°C and subcultured every month.

### Culture medium

The medium for inoculum preparation (seed medium) contained the following components (g/L): sucrose 100, yeast extract 3, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 1, K<sub>2</sub>HPO<sub>4</sub> 2, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.4, NaCl 2.5 and FeSO<sub>4</sub>·7H<sub>2</sub>O 0.05, pH 7.0. The fermentation medium contained (g/L): sucrose 150, peptone 5, K<sub>2</sub>HPO<sub>4</sub> 7, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.4, NaCl 3 and FeSO<sub>4</sub>·7H<sub>2</sub>O 0.05. The medium was autoclaved for 20 min at 121°C.

### Culture method

All fermentation experiments in our study, the seed culture of *A. pullulans* CGMCC7055 was

prepared by inoculating cells grown on potato-sucrose agar into 500 mL Erlenmeyer flasks containing 80 mL of seed culture medium, followed by incubation at 28°C for 29-31h in the rotary shaker (HYG-A, Taicang Experimental Equipment Factory, China) at 180 rpm.

The seed was cultured in the Erlenmeyer flask each time. When its absorbance measured at 600 nm using a spectrophotometer (UV-1800PC, China), was at 0.2-0.3, the seed would be transferred to the bioreactor (Shanghai, China) containing 3.5 L of the fermentation culture medium.

In order to investigate the effects of pH on cell growth and the yield of pullulan production without DO control, the pH of the medium was detected with an online pH probe (405-DPAS-SC K8S/325, Mettler Toledo, Switzerland). The effect of the pH was evaluated by controlling the pH at 2.0, 3.0, 4.0, 5.0, and 6.0 using automatic addition of 3M NaOH or 3M HCl during the course of the fermentation. The fermentation with the pH control was carried out at 28°C, 400rpm, and 1.0vvm for 88h.

As for DO, when it dropped to a set value, the effects of DO on cell growth and the production of pullulan were examined, the stirring speed was automatically increased and then decreased based on the value of DO (DO was cascaded to stirring speed through PID control), which was detected by an on-line DO probe (P52201018, S8238050, Mettler Toledo, Switzerland). The maximum oxygen saturation value achieved at aeration rate 7 L/min and stirring speed 700 rpm was calibrated as 100% of the DO probe. Then DO value detected by the DO probe in the fermentation process was the relative value compared with the calibration.

### Analysis methods

Samples were collected every 8 h for 88h and analyzed for biomass, yield of pullulan production, and residual sugar.

### Biomass

Five milliliter of broth was centrifuged at 2000g at 4 °C for 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°C overnight (>8h), dry weight was determined as biomass<sup>8</sup>.

### Determination of pullulan and assay of reducing sugar

Five milliliter fermentation broth was also centrifuged at 3300g at 4°C for 20 min (Super T-21, Sorvall Co., Norwich, CT). Four milliliter of supernatant was then mixed with 8 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°C until its weight was constant, then its dry weight was determined<sup>5, 13</sup>. The residual sugar concentration was measured in the cell free broth using Miller's method<sup>14</sup>.

## RESULTS AND DISCUSSION

### Effect of pH on cell growth and pullulan production

The pH levels during the fermentation process have the potential to influence the morphology of *A. pullulans* and in turn will also influence cell growth and pullulan production.<sup>9, 15</sup> Therefore, we investigated the effects of various pH levels, (ranging from 2.0 to 6.0 in 1.0 pH increments (controlled pH)), on cell growth and pullulan yield during CGMCC7055-induced

fermentation. Moreover, we also implemented the uncontrolled pH process, which started with an initial pH of 6.0 and fell gradually to 2.0 at the end of the fermentation. Results clearly show that regulating the pH during 88h of fermentation had a significant influence on cell growth as compared with uncontrolled fermentation (Fig. 1A, Table 1). For each controlled pH value specific growth rates ( $\mu$ ) increased with the passage of time during the adjustment stage, although we found that their growth rates were not the same. Moreover, results showed that the maximum specific growth rate had an optimal value of 0.21 h<sup>-1</sup> at pH 3.0 and that the maximum cell density ( $X_{max}$ ) had an optimal value of 13.2 g/L at pH 3.0. Interestingly, the  $X_{max}$  decreased as pH increased. Finally, the optimal cell yield ( $Y_{X/S}$ ) (0.088 g/g) and the optimal cell production rate ( $Q_x$ ) (0.63 g/L h) were both found to occur at a pH of 3.0 and to also decrease with increasing pH (Table 1). Taken together, these results clearly show that the optimal pH for pullulan cell growth is 3.0 and that higher pHs are detrimental to cell formation.

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

Different pH controlled processes	$\mu^a$ (h <sup>-1</sup> )	$Q_x^b$ (g/L h)	$Q_p^c$ (g/L h)	$X_{max}^d$ (g/L)	$P_{max}^e$ (g/L)	$Y_{P/X}^f$ (g/g)	$Y_{X/S}^g$ (g/g)	$Y_{P/S}^h$ (g/g)
Controlled at pH 2.0	0.20	0.43	1.34	12.00	38.17	3.18	0.081	0.30
Controlled at pH 3.0	0.21	0.63	2.38	13.20	50.53	3.83	0.088	0.34
Controlled at pH 4.0	0.19	0.29	2.09	11.67	61.00	5.23	0.082	0.29
Controlled at pH 5.0	0.18	0.25	2.42	11.22	67.53	6.02	0.075	0.45
Controlled at pH 6.0	0.17	0.20	1.29	10.79	44.22	4.10	0.071	0.31
Initial pH 6.0 (uncontrolled)	0.11	0.19	2.25	10.11	55.3	5.47	0.069	0.36

**Table 2.** Fermentation parameters of the batch experiments under various DO controlled processes in a stirred tank fermenter

Different pH controlled processes	$\mu^a$ (h <sup>-1</sup> )	$Q_x^b$ (g/L h)	$Q_p^c$ (g/L h)	$X_{max}^d$ (g/L)	$P_{max}^e$ (g/L)	$Y_{P/X}^f$ (g/g)	$Y_{X/S}^g$ (g/g)	$Y_{P/S}^h$ (g/g)
Controlled at DO 10%	0.13	0.38	1.25	9.46	48.22	5.10	0.067	0.34
Controlled at DO 30%	0.24	0.41	2.25	11.43	61.31	5.36	0.077	0.41
Controlled at DO 50%	0.25	0.42	0.63	12.77	25.47	1.99	0.088	0.17
Controlled at DO 70%	0.20	0.35	1.88	11.09	53.54	4.83	0.083	0.38
Uncontrolled DO	0.12	0.28	1.01	8.11	41.33	5.47	0.065	0.33

<sup>a</sup>The specific growth rate (?)

<sup>c</sup>The maximum pullulan concentration (P max)

<sup>e</sup>The optimal production formation rate (QP)

<sup>h</sup>The optimal cell yield (YX/S)

<sup>b</sup>The maximum cell density (X max)

<sup>d</sup>The optimal cell production rate (QX)

<sup>f</sup>The optimal specific product yield (YP/X)

<sup>g</sup>The optimal product yield (YP/S)

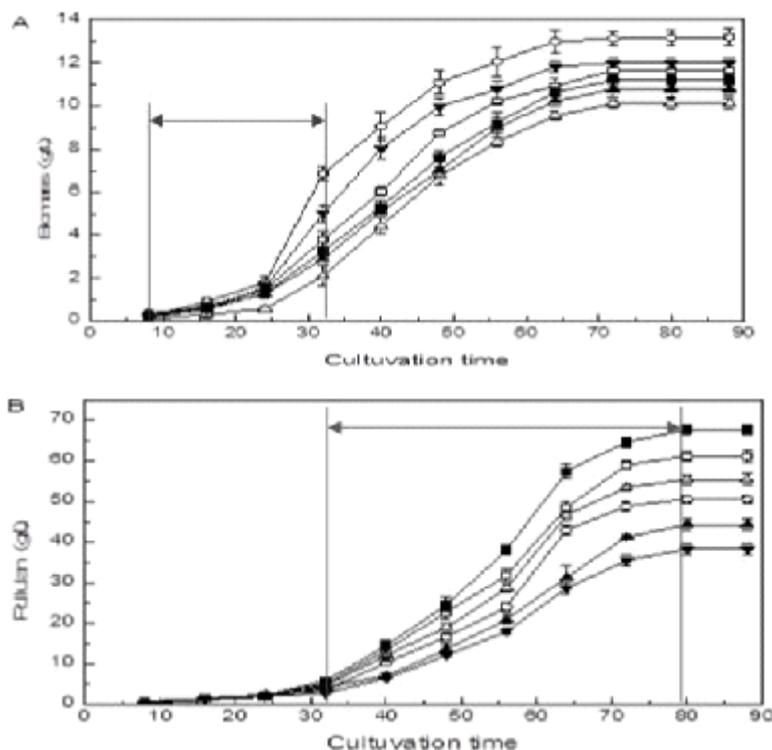
The influence of culture pH on the biosynthesis of pullulan was also significant and it was essential to elucidate the pH effects on pullulan production using the pH controlled processes compared with the pH uncontrolled processes as indicated in figure 1B and table 1. Pullulan began to be synthesized largely in middle and later stage (Fig.1 B). At pH 5.0, although cell growth is not the best, pullulan produced far ahead in all fermentation processes (Fig.1 B). And the maximum pullulan concentration (P max) of each pH controlled fermentation showed an optimal value 67.53 g/L at pH 5.0, and decreased by 43.5% at pH 2.0, decreased by 25.2% at pH 3.0, decreased by 9.7% at pH 4.0, and decreased by 34.5% at pH 6.0. While culture pH was uncontrolled, it fell gradually from 6.0 to 2.0, in comparison to the control fermentation, pullulan production was not the lowest. Like-wise, the optimal specific product yield ( $Y_{p/X}$ ) was 6.02 g/g obtained at pH 5.0. The optimal product yield ( $Y_{p/S}$ ) and the optimal production formation rate ( $Q_p$ ) also occurred at pH 5.0 were 0.45 g/g and 2.42 g/L.h, respectively. Higher

or lower culture pH than 5.0 inhibited pullulan formation.

Results indicated that relatively low pH (3.0) was suitable for cell growth, and relatively high pH (5.0) could promote pullulan production.

#### Effect of controlled DO on cell growth and pullulan production

During the process of pullulan fermentation, DO was one of the most important environmental factors affecting cell growth and pullulan biosynthesis. There had been many reports on the effects of DO on the production of microbial polysaccharides. The fermentation production of xanthan by *Xanthomonas campestris* was increased by increasing agitation speed<sup>16</sup>. Production of pullulan by *Aureobasidium pullulans* was also improved with an increase of impeller speed<sup>17</sup>. However, formation of scleroglucan by *Sclerotium glaucum* was found to be stimulated by DO limitation<sup>18</sup>. Thus, it appeared that the effects of DO on the production of various polysaccharides were different among different producing microorganisms. Therefore, it

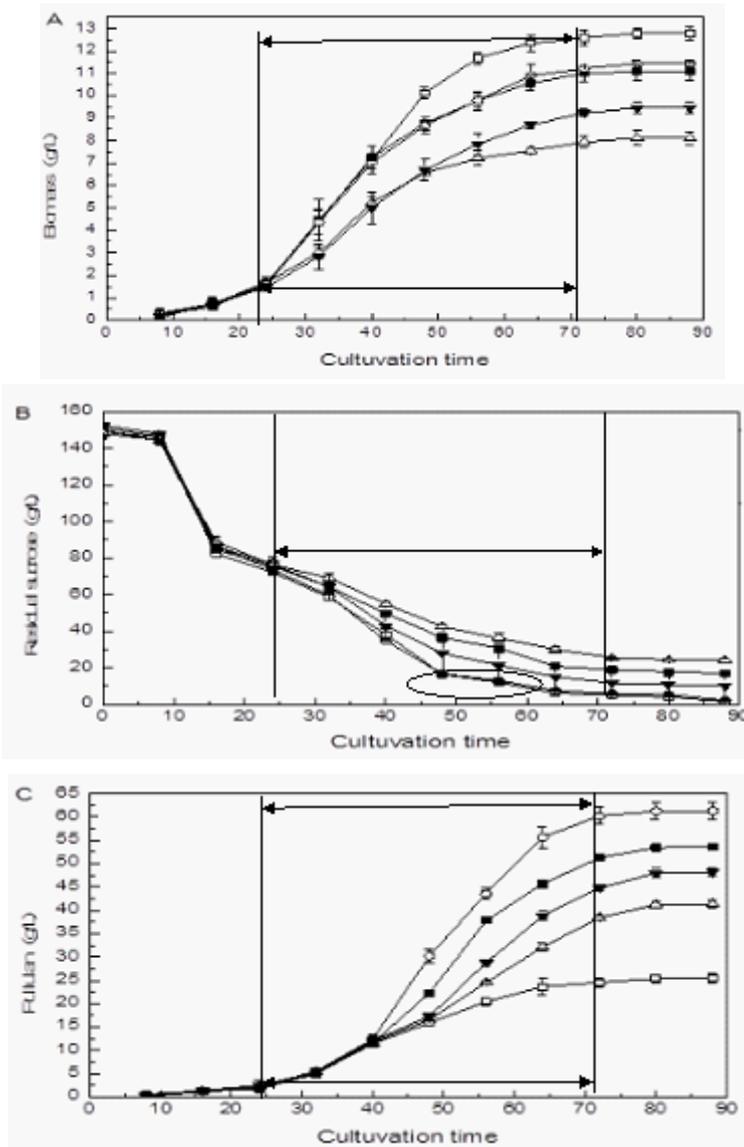


**Fig. 1.** Effect of controlled pH on cell growth (A) and pullulan production (B). Fermentation conditions: temperature, 28 °C; Stirring speed, 400 r/min; Aeration rate, 4 L/min; time, 88 h. Uncontrolled pH ( $\Delta$ ), pH 2.0 ( $\blacktriangledown$ ), pH 3.0 ( $\circ$ ), pH 4.0 ( $\square$ ), pH 5.0 ( $\blacksquare$ ), pH 6.0 ( $\blacktriangle$ ). Data are shown as mean  $\pm$  SD (n = 3).

was necessary to study the effect of DO on pullulan production by *A. pullulans* CGMCC7055. In our study, the influence of DO concentrations (10%, 30%, 50%, and 70%) and an uncontrolled DO processes on cell growth, residual sucrose concentration, and pullulan production were investigated. While the culture DO was uncontrolled, it fell gradually from 100% to 0, due to the increase of the liquid viscosity resulting

from the accumulation of extracellular pullulan. The results were shown in figure 2 and table 2.

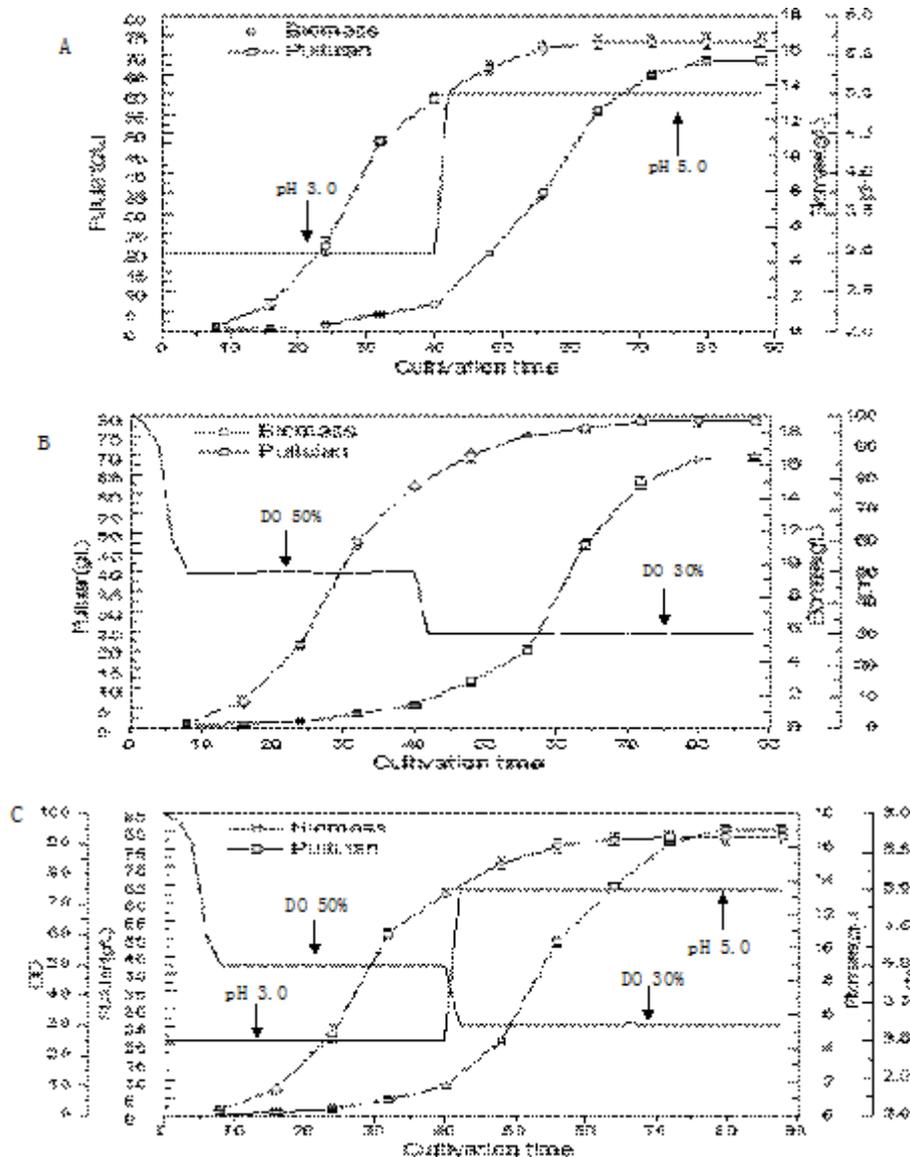
Under various DO concentrations, the kinetics of cell growth and pullulan production were different (Fig. 2A and C). However, the change of residual sucrose (Fig. 2B) was very similar during the entire fermentation process. In the adjustment stage, it could be seen from figure. 2 that the processes of each DO controlled fermentation were



**Fig. 2.** Effect of DO on cell growth (A), residual sucrose concentration (B), and pullulan production (C). Fermentation conditions: temperature, 28°C; Uncontrolled pH (initial pH6.0); time, 88h. Uncontrolled DO ( $\Delta$ ), DO 10% ( $\blacktriangledown$ ), DO 30% ( $\circ$ ), DO 50% ( $\square$ ), DO 70% ( $\blacksquare$ ). Data are shown as mean  $\pm$  SD (n = 3).

all similar due to the DO was not the limiting condition. After, the processes were all different. The maximum biomass (12.77g/L) was obtained at 50% DO condition. When the DO concentration was controlled at 70%, the cell growth decreased. It was indicated that a higher DO level was harmful to cell growth. These results on pullulan biosynthesis suggested that under low (10%) and

high (70%) DO concentration, pullulan production was reduced to 48.22g/L and 53.54g/L respectively. In addition, under 50% DO, pullulan production was the lowest (25.47 g/L), compared with the pullulan yield at other DO levels studied. The highest level of pullulan production (61.31 g/L) was achieved under the condition of 30% DO at 88 h (Table 2). However, at comparatively high DO



**Fig. 3.** Two-stage batch fermentation process for pullulan production. Figure 3A was a two-stage controlled pH (initially pH 3.0 and subsequently 5.0) with uncontrolled DO. Figure 3B was a two-stage DO (initially 50% and subsequently 30%) with uncontrolled pH (initial pH 6.0). Figure 3C was a two-stage pH combined with a two-stage DO. Data are shown as mean  $\pm$  SD (n = 3).

concentration (30% DO and 50% DO), the sucrose consumption was similar, faster than these at other DO levels studied (Fig.2B). It was indicated that sucrose was mostly used for pullulan biosynthesis under 30% DO, while under 50% DO, sucrose was mostly used for cell growth.

In a word, a comparatively high DO (50%) is beneficial for the cell growth and sucrose consumption in early stage, while a comparatively low DO (30%) is favorable for pullulan synthesis in middle and later stage.

#### **Two-stage batch fermentation process for pullulan production**

Previous studies had shown that the pH of 3.0 was beneficial for cell growth, but pH 5.0 was favorable for pullulan formation. Regarding the DO, 50% was beneficial for cell growth and 30% was beneficial for pullulan synthesis. Based on the fact that optimal pH and DO of cell growth is not in accordance with that of pullulan production, a novel control strategy of two-stage batch fermentation process was developed.

In this study, three types of two-stage fermentation were designed to be carried out: Type 1 was a two-stage controlled pH (initially pH 3.0 and subsequently 5.0) with uncontrolled DO fermentation (Fig.3A), Type 2 was a two-stage DO (initially 50% and subsequently 30%) with uncontrolled pH (initial pH 6.0) fermentation (Fig.3B). Type 3 was a two-stage pH combined with a two-stage DO fermentation (Fig. 3C). Fermentation conditions of three types were all changed at 40h.

Of all the three two-stage types of fermentation, the type 3 of fermentation stimulated the highest yield of pullulan (80.67 g/L) at a high cell density of 16.63g/L. and was 17.59% (w/w) and 16.07% (w/w) more than those achieved at two-stage pH (initially pH 3.0 and subsequently 5.0) with uncontrolled DO, and uncontrolled pH (initial pH 6.0) with two-stage DO (initially 50% and subsequently 30% ).

#### **CONCLUSIONS**

The studies had shown that the pH of 3.0 was beneficial for *A. pullulans* CGMCC7055, and pH5.0 was favorable for pullulan formation. As for the DO, 50% was beneficial for cell growth and 30% was beneficial for pullulan synthesis. Based

on the fact, a two-stage pH and DO process that maximized pullulan formation had been successfully demonstrated. This new and improved pullulan production process methodology would 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.

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