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Improvement of fermentative production of exopolysaccharides from *Aureobasidium pullulans* under various conditions

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Abstract−The optimization of exopolysaccharide (EPS) production was investigated in the polymorphic fungal strain of *Aureobasidium pullulans* (KCTC 6081) with varying pH, nutrients concentration, and mixing parameters in batch fermentation condition. The maximum production of EPS (~7.5 g/L) was observed at pH 4, while optimum nutrient concentration of carbon (sucrose), nitrogen ($NaNO_3$), phosphorous (K_2HPO_4), and ascorbic acid was 50 g/L, 5 g/L, 1 g/L and 2 g/L, respectively. Interestingly, EPS productivity under non pH controlled fermentation conditions was 0.12 g/L/h with 400 rpm mixing, while under a controlled pH of 4, the EPS productivity was 0.21 g/L/h with 600 rpm, respectively. The fed-batch fermentation increased the EPS productivity up to 0.345 g/L/h with changing mixing conditions from 200 to 600 rpm and reached 47 g/L with 88% pullulan. Thus, pH and mixing were the key parameters for enhancing EPS production from *A. pullulans*. It is expected that these optimized parameters can be well used for enhanced industrial production of pullulan.

Keywords: Optimization, Fermentation, Exopolysaccharide, Agitation, Nutrients

INTRODUCTION

Exopolysaccharides (EPS) are high-molecular-weight biopolymers (cellulose, dextran, gellan, xanthan, acetan, pullulan, and alginate) secreted by microorganisms (bacteria, fungi and algae) into their surrounding environment [1,2]. EPS are composed of sugar residues and utilized in a host of practical applications in various food and pharmaceutical industries such as thickeners, stabilizers, emulsifiers, gelling agents, and water binding agents [3]. However, obtaining EPS from nature is difficult, especially in homogeneous quantity and quality, due to the lack of literature pertaining to optimized conditions. Generally, EPS production requires a diverse range of experimentation, implying the necessary scale-up from bench to pilot scale fermentations once optimal conditions have been identified.

There are various physicochemical factors that affect the fermentation of EPS production such as environmental pH, mixing speed, temperature, and nutrients. Varying these factors can optimize the potentially useful EPSs in sufficient quantities in the confined system. Pullulan, a commercially important EPS produced by *Aureobasidium pullulans* [4], contains maltoriose units which can be used for medicinal and food additive purposes. More specifically, pullulan has been used for coating, packaging, as a sizing agent, a starch replacer, cosmetic emulsions, and edible films [5,6]. However, *A. pullulans* is a polymorphic fungus (single cell and filamentous multicellular) and harbors difficulties for growing in a fermentation system for EPS production. During cultivation of *A. pullulans*, the medium shows non-Newtonian characteristics, which lead to a difficult fermentation process such as unstable mass transfer and heat transfer. Therefore, vigorous mixing is an important factor for *A. pullulans* growth and its EPS production [2]. According to Chang et al. [7], the pH of the culture media was also vital for the production of pullulan from *A. pullulans* in which acidic pH produces a negative effect of inhibiting pullulan synthesis while stimulating the reproduction of fungi and only accumulating biomass. However, the optimum pH and mixing conditions for pullulan synthesis were not clearly characterized with *A. pullulans*.

The concentrations of the major nutrients of carbon and nitrogen can be adjusted to enhance the production of EPS at particular ratios [4,8]. For instance, low concentrations of nitrogen (C/N=157) favor the production of EPSs, whereas high concentrations of nitrogen (C/N=40) favor the production of biomass [9]. Additionally, the essential nutrients of phosphate and ascorbic acid were found to be necessary for microorganism proliferation and its EPS production [4,10]. Phosphorus is a key element for low molecular substance accumulation in the cell [10], whereas ascorbic acid functions as an oxidative agent to prevent browning of the strain and for stimulation of polysaccharide production [4]. However, most investigative efforts to-date have focused mainly on EPS from bacteria. Hence, little information is available with respect to the optimal conditions for industrially significant EPS production from *A. pullulans*.

In spite of several decades of effort, the production of EPS by polymorphic fungi has constantly yielded a low payoff of primary

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and secondary metabolites due to biological and engineering limitations. However, this issue can be improved by investigating the influence of certain controlled factors in a fermentation system. Hence, we aimed to optimize the properly formulated suitable media for the production of EPS from polymorphic fungal biomass of *A. pullulans* in a fermentation system using various pH, mixing speeds, and nutrient concentrations.

MATERIALS AND METHODS

1. Microorganism and Media

The strain of *A. pullulans* (KCTC 6081) was obtained from the Korea Research Institute of Bioscience and Biotechnology (KRIBB) and maintained in a modified Czapeak's medium [11]. The following are the modified media concentrations: Sucrose 3%, $NaNO_3$ 0.1%, K_2HPO_4 0.1%, KCl 0.005%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, Vitamin-C 0.3% and pH 4.

2. Flask and Jar Fermentor Experiment

The optimization parameters of different pH and nutrient concentrations experiment were conducted in a 500 mL Erlenmeyer flask containing sterile 100 mL of modified Czapeak's medium. The medium was the same in all experiments except the specific controlled variation (Fig. 1). Inoculum of 24 h old *A. pullulans* was inoculated at 4% v/v and incubated in rotary shaking incubator at 30 °C at 150 rpm. To scale up the experiment, a 5 L jar fermentor (Korea fermentor, Korea) was used with 3 L working volume and added to 6.6% v/v inoculum maintaining the temperature at 30 °C. The pH was measured with a pH probe (Mettler Toledo, USA) and controlled with 0.2 N HCl, while airflow was maintained at 1 vvm and mixing between 200-800 rpm. Experiments were conducted separately for in each type of variation.

3. Quantification of EPS

Samples were collected (7 mL) at frequent intervals and centrifuged at 10,000 rpm for 15 min. The supernatant (5 mL) was collected and transferred to a 50 mL centrifuge tube and added to 15 mL ethanol. Then, the tube was vortexed until a white precipitate formed and centrifuged at 10,000 rpm for 10 min. Then, the precipitate was removed with a spatula and transferred to pre-weighed plate and dried at 105 °C until no further decrease in weight was observed.

4. Optical Density (OD) and Dry Cell Weight Analysis

The pelleted cells in the quantification of EPS were washed twice with distilled water and OD was observed at 600 nm using a spectrophotometer (Kontron Instruments, Switzerland). Then cells were collected by centrifuging at 10,000 rpm for 15 min and transferred to a pre-weighed plate for drying at 105 °C until no further decrease in weight was observed. Finally, the dry cell weight was calculated.

5. Quantification of Pullulan

EPS produced from *A. pullulans* were assayed for sensitivity to pullulanase to determine the pullulan content. Enzyme solution for reaction conditions of pullulanase (amylopectin 6-gluconohydrolase, 25 unit, Sigma co.) were as follows: 2 mL of buffer (50 mM sodium acetate-acetic acid, pH 5) was added to 5 units. A total of 100 mL buffer was used for the reaction. The reaction temperature was set at 25 °C for 24 hrs. After the reaction, enzyme was inactivated by heat treatment for 5 mins. Enzyme activation was compared in accordance with the disposition of laminarine and pullulan.

6. Statistical Analysis

Results of flask experiment included using at least three samples from which the arithmetic mean and standard error of the mean were

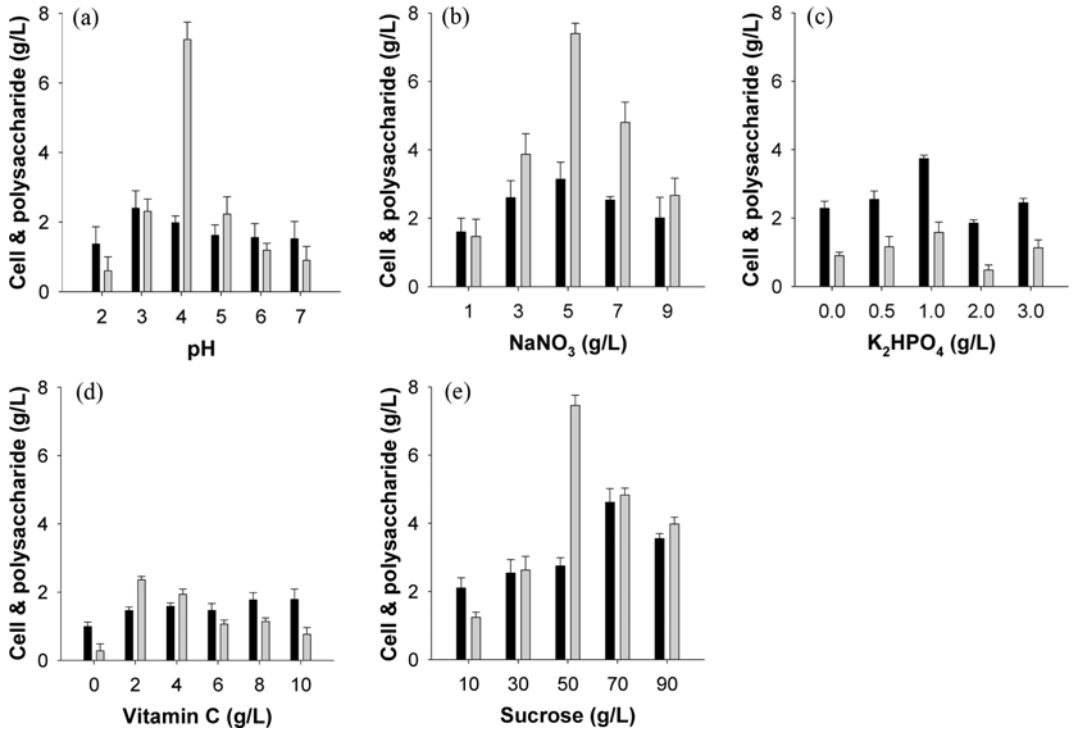

Fig. 1. The effect of variable (a) pH (b) $NaNO_3$ concentrations (c) K_2HPO_4 concentrations (d) vitamin C concentrations (e) sucrose concentration on the cell mass & polysaccharide productivity (■: DCW, ■: Polysaccharide).

calculated.

RESULTS AND DISCUSSION

1. Flask Experiment

1-1. Effects of pH

The pH of the media is one of the main factors affecting EPS production in *A. pullulans* [7]. Fig. 1(a) clearly shows the biomass and EPS production at various pH conditions from 2-7, which indicates that pH 4 was the optimum for EPS production (7.5 g/L) and pH 3 for biomass (2.5 g/L). This result was comparable to that provided by McNeil et al. [12] who stated that pH 4.5 induced the greatest pullulan production, while Simen et al. [13] noted that maximum production was reached at pH 5 from *A. pullulans*. This information clearly indicates that the optimum pH would slightly vary upon different strains and culture conditions. *A. pullulans* grows in the form of yeast or mycelium during growth with pH changes. Auer and Seviour [14] found a poor EPS production at pH 2.5 at which mycelium cells prevailed. However, at a higher pH the growth was not affected while yeast cells were dominant and EPS production was increased. Furthermore, Heald and Kristiansen [15] showed that as pH decreases (pH<4) the pullulan production decreases accordingly due to the reduction of yeast cell ratio. Since these studies claimed that EPSs are produced in yeast cells, we concluded that obtaining the optimal pH was of utmost importance for improving EPS production, which was found to be pH 4 according to our study.

1-2. Effects of Nitrogen and Phosphorous Concentration

It is well known that a high nitrogen concentration inhibits the production of EPSs in microbial synthesis, and that if nitrogen is depleted, EPSs are produced [14]. Therefore, different $NaNO_3$ concentrations (1-9 g/L) were used in the media compositions to observe their effects on EPS production (Fig. 1(b)). The maximum cell growth (3 g/L) and EPS production (7.5 g/L) was obtained at an optimum of 5 g/L $NaNO_3$, whereas high nitrogen concentrations were linked to a decreased EPS production. Similar results were discussed in previous related works by Gibbs and Seviour [16] and Auer and Seviour [14], who suggested that high concentrations (>5 g/L) of $NaNO_3$ hinder the production of EPSs. The optimum phosphate concentration for maximal EPSs production was noted to be 1 g/L (K_2HPO_4) (Fig. 1(c)). Furthermore, it was noted that biomass production was higher than the EPS production at any concentration of phosphorous. Therefore, the nutrient phosphate was not involved in EPSs production, but rather was important for biomass synthesis.

1-3. Effects of Ascorbic Acid

To prevent browning of the fungal strain and to induce EPSs production, various concentrations (0-10 g/L) of ascorbic acid (vitamin C) were used as an oxidizing agent, and the optimum conditions for EPSs production were observed. Fig. 1(d) reveals that 2 g/L of vitamin C was sufficient to enhance EPS production. On the other hand, withdrawal of vitamin C and adding more than 2 g/L showed less production of EPSs.

1-4. Effects of Carbon Source

Since the type of carbon source also affects the production of EPS, a selection of glucose, fructose, maltose, and sucrose was used in the production of EPS in the *A. pullulans* strain (data not shown). Among these sources, sucrose was linked to the highest productivity. Therefore, it was used as the carbon source in the downstream procedures in the optimization of EPSs production. A high concentration of sucrose (>50 g/L) inhibited the EPSs production. In contrast, the high concentration hindered the cell growth (Fig. 1(e)). However, maximum EPS production (~8 g/L) was noted at 50 g/L sucrose.

2. Jar Fermentation with and without pH Control

In azeotropic condition, higher fungal EPSs production can be achieved by way of optimizing the mixing speed, which increases the contact area between liquid and gas phases. In addition, the improved ventilation efficiency leads to enhanced EPS secretions. To investigate the mixing effect on EPSs productivity and cell growth, the experiment was conducted with various mixing speeds of 400,

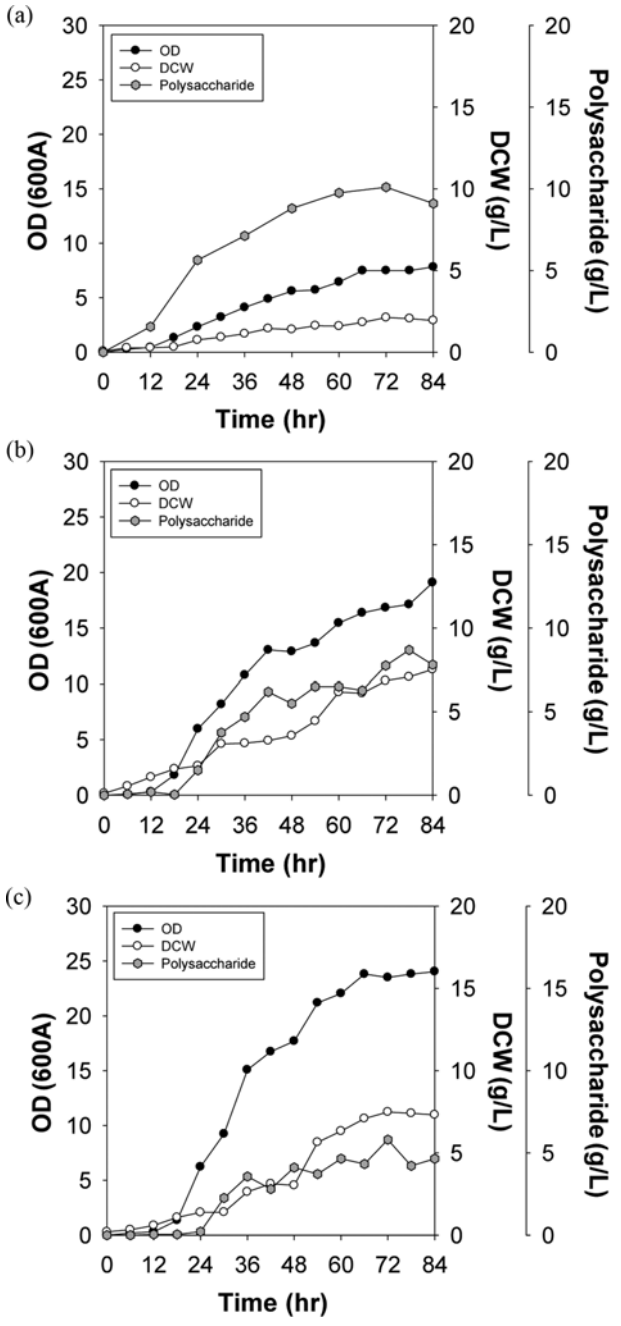

Fig. 2. Fermentation result with agitation speed (a) 400 rpm, (b) 600 rpm, (c) 800 rpm without control of pH.

600, and 800 rpm without a pH control and with an optimum pH control at 4. As a result, differences in cell growth were seen by varying the mixing speed, whereas EPS production did not show much difference without pH control sample (Fig. 2). However, at a fixed pH of 4, the production of EPS displayed significant differences with varying mixing speeds (Fig. 3).

As shown in Fig. 2, greater cell growth was observed while EPSs production decreased with increasing mixing speeds without a pH control system. Interestingly, the low rpm (400) was linked to the maximum EPSs at 10 g/L (yielded 0.12 g/L/hr) with 2 g/L cell growth, whereas in high rpm (800) ~5 g/L EPSs and ~7.5 g/L cell biomass were seen. Several reasons can be correlated to decreased EPS production, but the most notable factor was the final pH of the medium, notably the pH values at 5.45, 6.20, and 7.78 for mixing speed of 400, 600, and 800 rpm, respectively. However, this study revealed that *A. pullulans* produced maximum EPSs at pH 4. In addition, the end pH was increased >4 at which decreased production of EPS was noticed. As discussed in previous studies [18], this result also supported the finding that pH is the most important factor for EPSs production in *A. pullulans*.

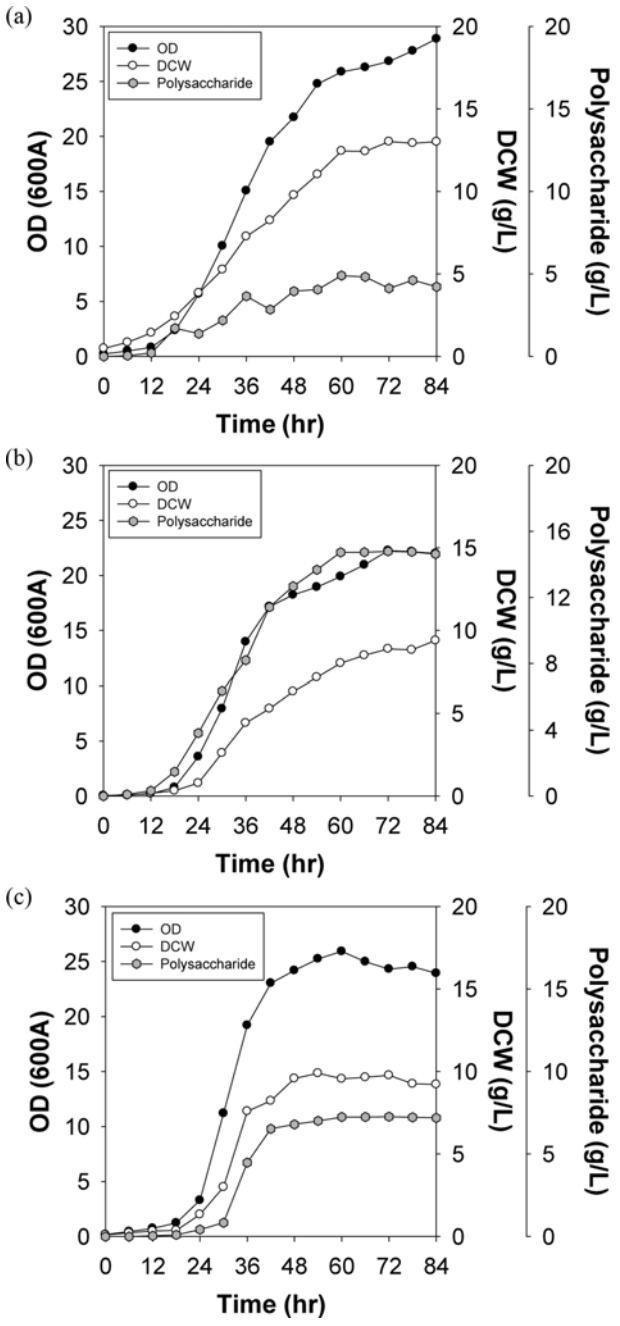

Fig. 3. Fermentation result with agitation speed (a) 400 rpm, (b) 600 rpm, (c) 800 rpm under the control of pH at 4.

Accordingly, the fixed optimum pH experiment was also conducted with different mixing speeds and clearly showed the significance of pH for EPS production (Fig. 3). The EPSs reached 15 g/L with a yield of 0.21 g/L/hr at 600 rpm and cell growth attained at 10 g/L. However, the mixing speed at 400 rpm yielded only ~5 g/L EPSs with a maximum of 13 g/L cell growth. On the other hand, the maximum speed of 800 rpm displayed comparatively less EPS than 600 rpm, which might have been the result of the shear stress breaking the cell and destroying the mycobiont [19]. These findings revealed the importance of mixing speed on EPS production. In this case, it was found to be 600 rpm. Some previous studies suggested the idea that the aeration was crucial for EPS production [20] and was closely related to the mixing speed. Furthermore, these works also suggested that despite having 1.5 times higher cell mass in 400 rpm than any other mixing speed but observed low production of EPS, there is no direct proportional relationship between cell mass and EPS productivity.

3. Fed-batch Fermentation

Previous studies have reported the contradicting effects of mixing speed such as the claim that higher mixing speed leads to better aeration and increases yeast-like cells and EPSs production. Furthermore, others have claimed that lower mixing speed maintains dissolved oxygen, which is directly related to increasing yeast-like cells and EPSs [12,21]. Therefore, in this study, mixing speed was changed from low (200 rpm) to high (600 rpm) during initial stage and sucrose was fed twice in the middle and later stages of cultivation (Fig. 4). The production of EPSs and cells was increased up to ~48 g/L and 18 g/L, respectively, on the 5th day of incubation which was comparable to the findings of a previous study at 50 g EPS/L [21]. Fed-batch cultivation led to productivity at 0.345 g EPS/L/hr

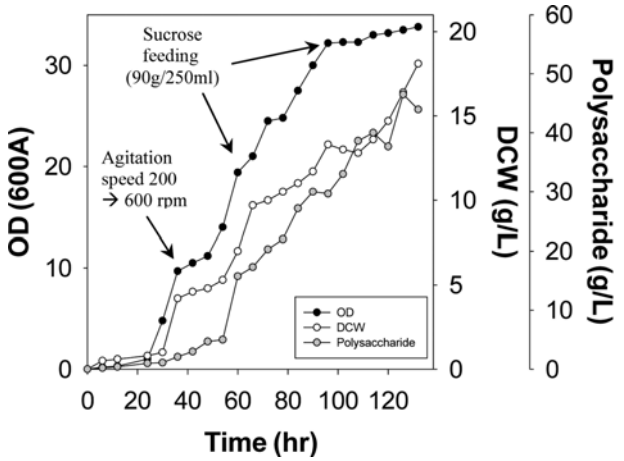

Fig. 4. Fed-batch fermentation result with twice feeding and agitation speed change from 200 to 600 under the control of pH at 4.

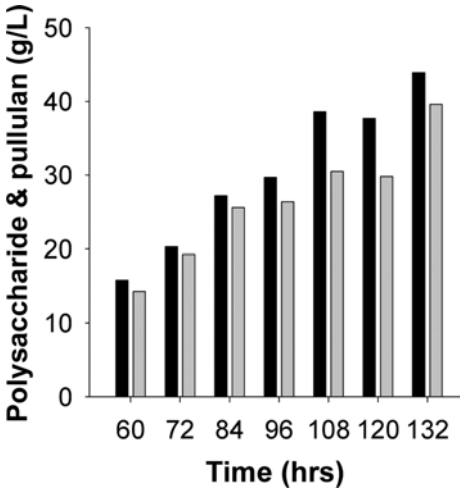

Fig. 5. Pullulan production from Fed-batch fermentation (■: Polysaccharide, ■: Pulluan).

and also attained 1.64-times higher productivity and 3.2-times more production than that produced by batch fermentation with 600 rpm. Moreover, pullulan content constituted 88% of the total EPSs produced through fed-batch fermentation (Fig. 5). Hence, we suggest that the EPSs production could be enhanced by fed-batch cultivation.

CONCLUSION

EPSs production in *A. pullulans* obliges optimal culture condition and must be cost effective for scale-up to industrial production. Hence, optimization was achieved through varying key factors including pH, nitrogen, phosphorous, carbon, ascorbic acid, and mixing speed. The optimal pH for maximum production of EPSs was found to be 4, while optimal concentration for nitrogen, phosphorous, carbon, and ascorbic acid were 5 g/L, 1 g/L, 50 g/L and 2 g/L, respectively. Furthermore, the ideal mixing speed without pH control was 400 rpm, and with pH control at 4 was 600 rpm, which resulted in maximum productivity of 0.12 g/L/hr and 0.21 g/L/hr EPSs, respectively. Finally, the condition with changing mixing speed in fed-batch fermentation yielded a productivity maximum of up to 0.345 g/L/hr EPS with 88% pullulan. These optimized parameters demonstrate high potential for pullulan production and can be well used for commercialization of pullulan.

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