

Study on the submerged fermentation process producing perylenequinones derivatives by *shiraia* sp. AL18

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Abstract. The perylenequinones derivatives (PQDs) are potential photosensitizers, which have a high effective light-induced biological activity, such as anti-HIV, antimicrobial and anticancer activities. In this work, the submerged fermentation process of filamentous fungus *shiraia* sp. AL18 producing PQDs in a stirring fermentation tank was investigated through single factor optimization. The effect of the stirring speed (450 r/min, 500 r/min and 550 r/min at ventilation quantity for volumes of air per volume of liquid per minute (VVM) = 1:1) and ventilation quantity (VVM = 1:0.8, 1:0.65 and 1:0.5 at stirring speed of 500 r/min) on the process of submerged fermentation by stirring fermentation tank in batch fermentation was investigated using the monosyllabic test. The results suggest that the optimum stirring speed for biomass is 500 r/min and the optimum ventilation quantity for biomass is VVM = 1:0.65, and the maximum dry cell weight (DCW) reached to 19.67 g/L. The stirring speed for the optimum yield of PQDs is 500 rpm and the ventilation quantity for the optimum yield of PQDs is VVM=1: 0.8, and the maximum yield of PQDs reached to 0.3218 g/L. The yield of PQDs was correlated with pH value, dissolved oxygen (DO) value, the content of reduced sugar in the period of fermentation.

1 INTRODUCTION

The perylenequinones derivatives (PQDs) are a class of compounds characterized by a pentacyclic conjugated chromophore of the 4,9-dihydroxy-3,10 perylenequinoid group (1, 2). A few PQDs such as Hypocrellins A (HA), Hypocrellins B (HB), Elsinochrome C (EC) and Cercosporin are potential photosensitizers, have a high effective light-induced biological activity, such as anti-HIV and anticancer activities (3-6). PQDs not only can be used in next generation photodynamics therapy (PDT) medicine, but can also be extensively used for photo dynamic inactivation of gram-positive bacteria, various fungal pathogens and *Bursaphelenchus xylophilus*, etc.(7, 8). Since their biological activity depends on the production of oxygen free radical induced by light wavelength and intensity selectivity, and free radical can attack biological macromolecules in multiple targets, PQDs have a natural advantage in dealing with the problem of superbugs resistance. Although PQDs were once considered ineffective in inhibiting gram-negative bacteria such as *Escherichia coli*, it had been confirmed that their bacteriostatic activity significantly improved by adding lanthanide ions or bivalent cations such as CaCl_2 or MgCl_2 (9, 10). Given that PQDs have many applications in the fields of medicine, disinfection and preservation, it is important to achieve industrial scale production of PQDs as soon as possible. Traditionally, several PQDs (especially HA, HB and EC) are extracted from the stromata of *Hypocrella bambusae* and *Shiraia*

bambusicola (11, 12). However, this approach is limited by natural resources, as the stromata mentioned above are actually parasitic fungi of bamboo which produce pathogens of bamboo diseases. Moreover, PQDs like HA can be totally synthesized in 19 steps, but the overall yield is only 1.6% (13), thus its synthesis by such method is not economic nor realistic in large scale applications of PQDs. Recently, several endophytic fungi were isolated, identified and classified as anamorphs of *shiraia* sp., which can produce PQDs in solid fermentation or shaking flask fermentation (14-17). After optimization of the culture medium and conditions, the maximum yield of PQDs reach to 4.7 mg/g, 102.6mg/L, 196.94 mg/L, and 1.21 g/L, respectively (14, 18-20). Although previous research opened up the possibility for the large-scale application of PQDs, but solid fermentation has the natural defects in small production scale, which lead to long fermentation cycle and low extraction efficiency. So, the submerged fermentation process for stirring fermentation tank were studied in order to realize industrial production of PQDs.

2 MATERIALS AND METHODS

2.1 Strain, medium and seed culture

The strain used in this paper is *shiraia* sp. AL18, a stock culture from the fermentation engineering laboratory, School of Life Science, Shandong University of

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Technology, Zibo, Shandong province, P. R. China. The inter-transcribed spacer (ITS) sequence of strain AL18 (GenBank accession number AY425966) was identified and classified as *shiraia* sp. according to a Blast and cluster analysis (21). The slant-medium used is photo-dextrose agar (PDA), the small pieces of mycelia were inoculated on slants and cultured in the dark at 28°C for 5 days. An integrated potato culture medium was used for seed culture, which is prepared by adding peptone (10 g), KH₂PO₄ (1 g), MgSO₄·7H₂O (0.5 g), Vitamin B1 (0.1 g) into one liter of photo-dextrose medium and autoclaving at 121°C for 20 min. Slant culture was cut into 4-5 pieces and inoculated into 70 mL of the above mentioned medium in a 250 mL flask, cultured at 28°C for 7 days. For seed sharking culture, the medium is based on the integrated potato culture medium, except that the content of peptone is changed from 10 g to 3 g, and extract of silkworm pupa meal (12.5 g) and CuSO₄·5H₂O (0.05 g) are also added. The fungal velum was sliced into fine pieces and equally inoculated into seven 70 mL sharking flasks, cultured at 28°C, at 180 r/min for 3 days.

2.2 Submerged fermentation process by stirring fermentation tank

Fermentation was carried out in a stirred-tank fermentor (10L, GUJS-10, ZheJiang East Biotech Equipment Co., Ltd, China), the content of the fermentation medium is the same as that of the seed sharking culture medium, with 0.1% SDS-10 (Nanjing Well Chemical Co., Ltd.) added as defoaming agent, the loading coefficient is 70%, and the initial pH was adjusted to 5.75 with 1 M NaOH before autoclaving at 121°C for 20 min. The inoculum size was 10% of the seed shark culture, the pressure and temperature of the tank was maintained at 0.05 MPa and 28°C by an automatic control system. The effect of the stirring speed (450 r/min, 500 r/min and 550 r/min at ventilation quantity for volumes of air per volume of liquid per minute (VVM) = 1:1) and ventilation quantity (VVM = 1:0.8, 1:0.65 and 1:0.5 at stirring speed of 500 r/min) on the process of submerged fermentation by stirring fermentation tank in batch fermentation was investigated using the monosyllabic test. The value of the pH and dissolved oxygen (DO) concentration (relative content) were recorded on-line by the corrected pH electrode and DO electrode in the period of fermentation, the fermentation liquid was also sampled at intervals of 4 h during the period of fermentation and biomass; the yield of PQDs and the content of total soluble sugar, reducing sugar, total protein and FAN were measured to investigate the relationship between the tendency of the pH, DO, substrate consumption, growth of the mycelia and accumulation of PQDs during the period of fermentation.

2.3 Analytical methods

A 20 mL sample was centrifuged at 4°C, 5000 r/min for 20 min, the supernatant was diluted up to 20 mL with distilled water and used for subsequent assays, the

content of total soluble sugar, reducing sugar, total protein and free amino nitrogen (FAN) were measured by the vitriol-phenol method, 3,5-dinitrosalicylic acid (DNS) assay, Lorry assay and formaldehyde titration method. The sediment was washed twice by centrifugation with 20 mL distilled water, and then the biomass was measured by the dry cell weight (DCW) method. The PQDs content was measured by spectrophotometry as described previously (11), dried mycelia was ground and extracted with 40 volumes of ethanol (w/v) by hot reflux for 30 min, the extraction was filtered using a G4 sand core funnel, the volume was measured, the filtrate was properly diluted and the absorbance was measured at 460 nm, the PQDs content was calculated according to the standard curve (taking HA as standard) and the regression equation $A = 0.0117 + 0.0458C$, where A is the absorbance at 460nm, C is the concentration of PQDs $\mu\text{g/mL}$ and $R^2 = 0.9956$. The content of the above biochemical components is reported as the mean of three measured values and the standard deviation.

3 RESULTS

3.1 The effect of the stirring speed and ventilation quantity on biomass and the yield of PQDs

The effect of the stirring speed on biomass and the yield of PQDs is shown in Fig. 1 A. In the period of fermentation, the DCW was slightly increased 4 h after inoculation, grew rapidly from 4 h to 32 h when it reached the maximum DCW, and declined from 32 h to 40 h. The DCW increased greatly from 12.56 g/L to a maximum of 17.50 g/L when the stirring speed increased from 450 r/min to 500 r/min, and then there was a small decline from 17.50 g/L to 16.38 g/L when the stirring speed increased from 500 r/min to 550 r/min. The production of PQDs was initiated at 20 h, fast-accumulated to reach the maximum yield between 12-16 h, and then decreased rapidly. The maximum yield of PQDs reached to 0.2039 g/L, 0.3012 g/L and 0.2876 g/L, these results indicated that increasing the stirring speed within an appropriate range will improve the yield of PQDs, but too high stirring speed will reduce the yield of PQDs. The effect of the ventilation quantity on biomass and the yield of PQDs is shown in Fig. 1 B. Increasing the VVM from 1:0.8 to 1:0.65, the DCW will increased from 17.27 g/L to the maximum of 19.67, but in the case when the VVM was increased to 1:0.5, the maximum DCW greatly declined to 16.09 g/L. The maximum yield of PQDs occurred earlier and then decreased from 0.3218 g/L to 0.2286 g/L accompanied with the increase of the ventilation quantity.

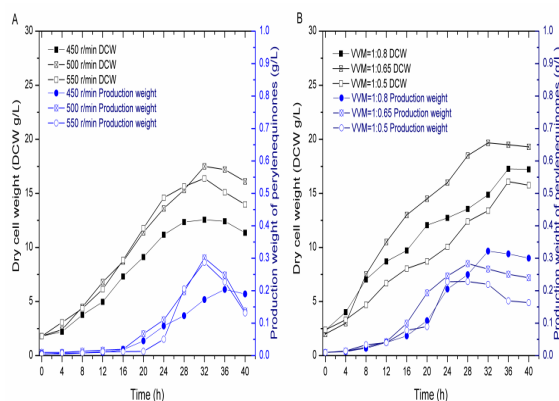


Fig. 1. The effect of the stirring speed and ventilation quantity on the biomass and the yield of PQDs. A: the effect of the stirring speed on cell growth and production of PQDs, B: the effect of the ventilation quantity on biomass and the yield of PQDs.

3.2 The effect of stirring speed and ventilation quantity on pH and DO curve

The trend of the pH and DO curve is shown in Fig. 2. The pH value and DO concentration of the fermentation liquid began to decline after inoculation and reached their lowest point, then return to their highest point and were maintained until the end of the fermentation period. Increasing the stirring speed from 450 r/min to 550 r/min, the lowest pH point occurred later and declined from 5.19 to 4.68, however, the pH value in the middle-later stage of fermentation was reduced from 6.19 to 5.73. Increasing the stirring speed from 450 r/min to 550 r/min, the lowest DO concentration point occurred earlier and the DO value in the middle-later stage of fermentation increased from 63.2% to 71.8%, as shown in Fig. 2A. The effect of the ventilation quantity on the pH curve was less pronounced (shown in Fig. 2B), the pH value in the middle-later stage of fermentation only rose from 5.94 to 6.29, accompanied with the increase of the ventilation quantity. The effect of the ventilation quantity on DO is shown as Fig. 2B, the lowest DO concentration point occurred slightly earlier and appeared slower compared to VVM = 1:0.8, the highest DO value in the middle-later stage of fermentation rose from 72.2% to 89.8%, increasing the ventilation quantity from VVM = 1:0.8 to VVM = 1:0.65. However, the highest DO value in the middle-later stage of fermentation declined from 89.8% to 88.7%, accompanied with the increase of the ventilation quantity from VVM = 1:0.65 to VVM = 1:0.5.

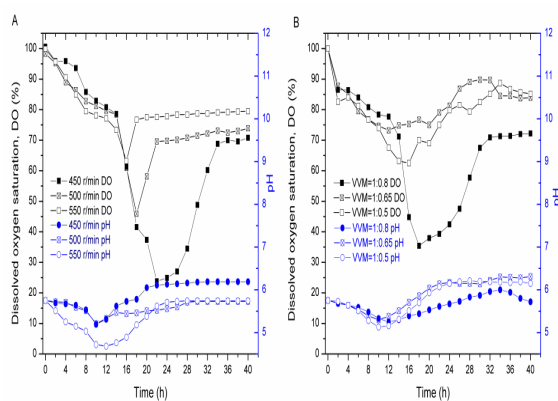


Fig. 2. The effect of the stirring speed and ventilation quantity on the pH and DO curve. A: the effect of the stirring speed on the pH and DO curve, B: the effect of the ventilation quantity on the pH and DO curve.

3.3 The effect of the stirring speed and ventilation quantity on substrate consumption

As shown in Fig. 3A, increasing the stirring speed reduced the total sugar consumption rate at 0-4 h and increased the total sugar consumption rate at 20-28 h in fermentation period, the peak content of reducing sugar occurred earlier and was maintained at a higher level in the next period of fermentation when the stirring speed increased from 450 to 500 r/min. When the stirring speed increased to 550 r/min, the content of reducing sugar was lower than that at 500 r/min. Regarding the effect of the ventilation quantity on total sugar consumption and the content curve of reducing sugar (shown in Fig. 3B), increasing the ventilation quantity from VVM = 1:0.8 to VVM = 1:0.65 reduced the total sugar consumption rate, whereas the total sugar consumption rate was increased by increasing the ventilation quantity from VVM = 1:0.65 to VVM = 1:0.5. However, the content of reducing sugar was declined compared to when the ventilation quantity was VVM = 1:0.8.

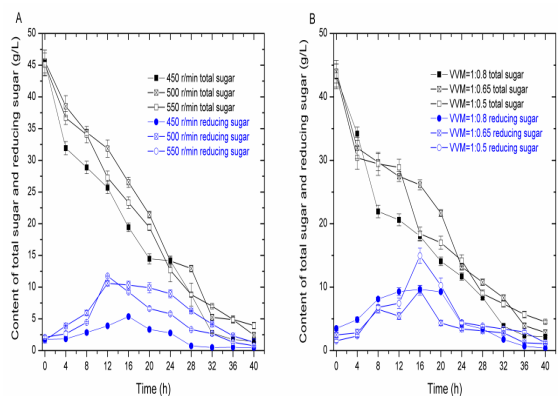


Fig. 3. The effect of the stirring speed and the ventilation quantity on the content curves of total sugar and reducing sugar. A: the effect of the stirring speed on the content curve of the total sugar and reducing sugar, B: the effect of the ventilation quantity on the content of total sugar and reducing sugar.

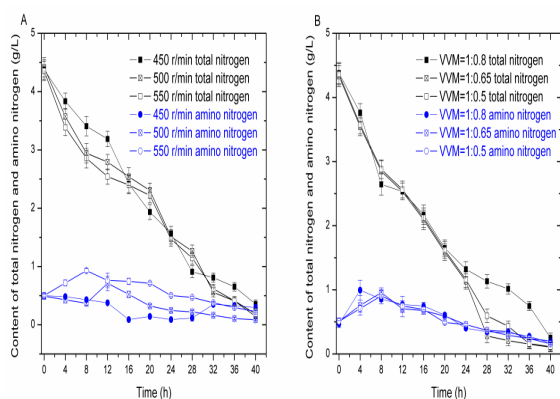


Fig. 4. The effect of the stirring speed and ventilation quantity on the content curve of total nitrogen and FAN. A: the effect of the stirring speed on the content curve of total nitrogen and FAN, B: the effect of the ventilation quantity on the content of total nitrogen and FAN.

The effects of the stirring speed on nitrogen consumption and the content of FAN are shown in Fig. 4A. Compared with the stirring speed of 450 r/min, accompanied by increasing stirring speed, the total nitrogen consumption rate is increased at 0-12h, and decreased after 12h in the period of fermentation, and has higher total nitrogen utilization. Compared to stirring speed 450 r/min, increasing the stirring speed will increase the content of free FAN at 0-28 h, but the lowest content of FAN 0.105 ± 0.072 g/L appeared at 28-40h when the stirring speed reached to 500 r/min. The effects of the ventilation quantity on nitrogen consumption and the content of amino acids are shown in Fig. 4B. Compared to when the VVM = 1:0.8, there is no significant effect on the content of FAN accompanied by the increase of the ventilation quantity to VVM = 1:0.5, besides the content peak of FAN appeared later, and has higher total nitrogen consumption rate after 20 h in the period of fermentation.

4 DISCUSSION

In this work, the effect of the stirring speed and ventilation quantity on biomass, yield of PQDs, pH, DO, total sugar, reduced sugar, total nitrogen and FAN were investigated. The results show that a pH value at about 5.75, appropriate improvement of the DO value and a higher concentration of reduce sugar are beneficial to the production and accumulation of PQDs in the middle-later stage of the fermentation period. The PQDs yield was significantly lower than that previously reported in the shake flask process (20, 22). It is a plausible speculated that the excessive DO level inhibited the synthesis of PQDs possibly due to the adverse effect of oxidative stress on metabolism of the mycelin caused by oxygen free radicals which can be produced by PQDs (23), but the results of oxygen stress improved the yield of hypocrellin A make the conjecture become suspicious (24). A reasonable explanation is that the decline of the yield of PQDs possibly due to the effect of the mycelial morphology, physiology and the damage of the agitator under the conditions of the stirring fermentation tank (25-27). A genetically-engineered strain carrying an

hemoglobin gene from *vitreoscilla* sp. (VHB) may reduce the mechanical damage and guaranteed the supply of DO at lower stirring speed. The high concentration of reduced sugar may improve the source of carbon skeleton, and is beneficial for the PQDs synthesis, which suggests that using a single carbon source, such as sucrose or glucose, and controlling the concentration of reduced sugar in the fermentation may greatly increase the yield of PQDs.

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