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Cold Plasma Pretreatment Promoting Penicillin Accumulation in Penicillium Chrysogenum

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Abstract: *Penicillin is the most important class of β-lactam antibiotics. The improvement of high-yielding strain has always been the focus of low-cost industrial production. In this study, Penicillium chrysogenum CICC 40654 was pretreated by helium cold plasma, and then used for fermentation to synthesize penicillin. Through single factor test and orthogonal one, the optimal values of pretreatment parameters such as discharge power, discharge time and helium working pressure were discussed. Results showed that the strain pretreated at 130 W and 100 Pa for 35 s had the highest penicillin synthesis (more than 30 μg/g dried mycelium biomass) on fermentation for 3 days, which was about 79.7% higher than that of the control. In summary, green and efficient cold plasma pretreatment enhanced its ability of penicillin accumulation of Penicillium chrysogenum. This study will provide a new methodological basis for improving industrial strains for high-yield biomedicine.*

Keywords: *Cold plasma, Penicillin, Penicillium chrysogenum*

1. Introduction

Penicillin, a kind of broad-spectrum β-lactam antibiotic, was a milestone in the history of human medicine. It has been widely applied to clinical treatment, such as the therapy of sepsis, pneumonia and other bacteria infective diseases [1-4]. However, the comparatively low antibiotic titer in the fermentation became the bottleneck for industrial production [5,6].

So far, enormous efforts in classical strain improvement (CSI) programs including random mutagenesis, medium and process optimization, and genetic engineering were carried out for achieving high-titer penicillin production [7-10]. Weber et al., for example, reported overexpression of one penicillin biosynthetic gene PenDE that encoded isopenicillin N acyltransferase (IAT) in Penicillium chrysogenum (P. chrysogenum). The genetically engineered strain showed a significant increase in penicillin V production as high as 160-200% [7]. However, expensive cost, complicated operation and long development cycle for above strain improvement programs also restricted the antibiotic supply at lower prices. Obviously, developing a simple, efficient and green strain improvement approach is necessitating for industrial antibiotic production.

Plasma, the aggregation of ions, photons, excited atoms, free electrons and radicals, has attracted wide attention in the past decade in the fields of medicine, food processing, material surface modification and especially microbial strain improvement [11-13] due to the strong interaction between plasmas (including high-energy particles and radiations) and organisms. Compared to CSI methods, cold plasma pretreatment approach has the characteristics of green, convenience, low cost, safety, high efficiency and controllability [14-16]. In our previous study [17], Bacillus amyloliquefaciens pretreated by cold plasma showed enhanced α-amylase secretion and enzyme activity in the fermentation. But it was unclear for the effects of cold plasma on secondary metabolites.

In this study, Penicillium chrysogenum CICC 40654 (P. chrysogenum) was pretreated through radio-frequency (RF) cold plasma under low vacuum and room temperature, using helium as plasma source, to increase penicillin accumulation in the fermentation. The optimal pretreatment parameters including discharge power, working pressure and pretreatment time were established successively through single factor assays and orthogonal experiments. This work developed a simple and green strain improvement approach for industrial antibiotic production, and further understanding the impact of cold plasma on secondary metabolites.

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2. Materials and methods

2.1 Strain, culture and cold plasma pretreatment

P. chrysogenum CICC 40654 purchased from China Center of Industrial Culture Collection (CICC) was cultured in Potato dextrose agar (PDA) medium containing 5.0 g/L potato powder, 20.0 g/L glucose, 15.0 g/L agar (pH5.8-6.2) [18]. The lipid PDA medium without agar was also used as fermentation medium.

A cold plasma device (HD-2N, Changzhou Zhongke Changtai Plasma Tech. Co., Ltd, China) equipped with a 13.56 MHz radio-frequency (RF) generator is used for treating P. chrysogenum with helium as the plasma source [19]. The treatment conditions were as follows: discharge power 100-180 W, working pressure 70-150 Pa, and pretreatment time 10-50 s. All the tests were conducted in triplicate.

2.2 Aminobenzylpenicillin fermentation of P. chrysogenum

Cold plasma pretreated P. chrysogenum was inoculated to 100 ml PDA medium for fermentation in 250 mL flask at 200 r/min and 30 ℃ for 5 days [20,21]. Take the samples from the 3rd day of fermentation for analysis of penicillin accumulation [22]. Mycelia were collected from the samples and dried to a constant weight (45 ℃ overnight) for calculating the mycelia biomass. Supernatants were further used for aminobenzylpenicillin extraction. All assays were carried out in replicates. Untreated strain was used as the control.

2.3 Extraction of aminobenzyl penicillin from P. chrysogenum

A 6 mL fermentation supernatant was transferred to a 10 mL tube after centrifuged at 8000 r/min for 5 min. The supernatants were acidified with 0.1 N HCl until pH 2.0. Benzylpenicillin was extracted by adding n-butyl acetate (3 × 2 mL) and re-extracted from the organic phase with 10 mM phosphate buffer pH 7.5 (3 × 2 mL) at 4 °C. The aqueous phase lyophilized and re-suspended in 200 μL of Milli-Q water for filtrates was used for the future analysis [22].

2.4 HPLC analysis

Benzylpenicillin was determined using the Agilent 1100 HPLC system (Santa Clara, CA, USA) with an analytical 4.6 × 250 mm (5 μm) RPC18 Lichrospher® 100 (Merck, Darmstadt, Germany) column at 1.0 mL/min and 214 nm. A 20 μl sample filtered with 0.22 μm was injected and eluted using as mobile phase buffer A (30 mM ammonium formate pH 5.0 and 5% acetonitrile) and buffer B (same as buffer A plus acetonitrile 20:80, v/v) with an isocratic method (85% of A) [22].

3. Results and Discussion

3.1 Effects of cold plasma pretreatment on penicillin accumulation

Cold plasma pretreatment involved three parameters including discharge power, working pressure and pretreatment time. Their effects on mycelia growth and penicillin accumulation were studied here.

Firstly, different discharge power (100-180 W) was operated, and the working pressure and pretreatment time were kept unchanged, at 135 Pa and 15 s, as shown in Figure 1. Generally speaking, cold plasma pretreated strain has higher activity than the control. For example, the biomass of mycelium pretreated at 140 W reached about 0.99 g per100 ml of fermentation broth, which was 33.8% higher than that of the control (0.74 g) (Figure 1a). Similarly, the pretreated strain also showed stronger penicillin secretion ability than the control (Figure 1b). At 140 W, the penicillin secretion of the pretreated strain was 20.43 ± 0.42 μg/g biomass, while that from the control strain was only 18.25 ± 0.25 μg/g biomass, which was 10.7% lower than that of the pretreated strain. Results indicated that plasma generated in the discharge power range of 100-180 W had positive effects on the activity of the strain. Noted that in the five treated strains, the biomass and penicillin secretion reached the peak at 140 W, which was probably due to the plasma energy generated at different discharge powers. It is well known that the radiation and energy of the plasma steadily enhanced with the discharge power increased. Obviously, low discharge power (100-120 W) produced low level of radiation and energy, which had a small impact on the improvement of strain activity [23]. In contrast, the high radiation and energy might have a negative

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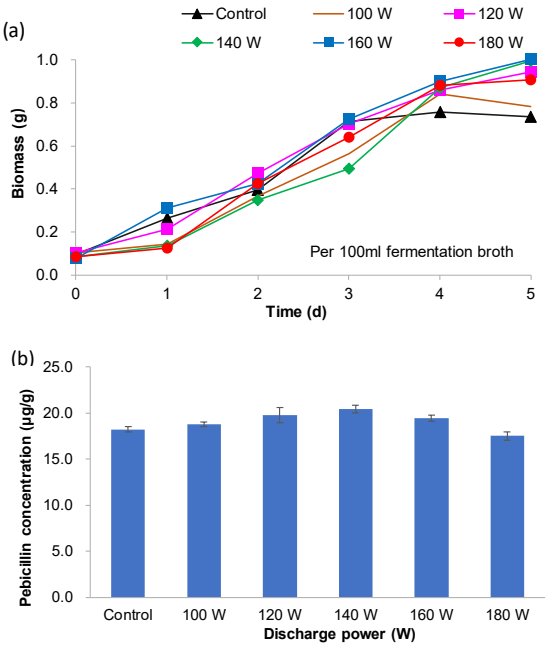
impact on cells, leading to decreased activity.



(a) mycelia growth; (b) penicillin concentration.

Figure 1: The effect of different discharge power of cold plasma on penicillin accumulation for P. chrysogenum.

After that, different pretreatment time (10-50 s) was operated, and the discharge power and working pressure were kept unchanged, at 120 W and 135 Pa (Figure 2). Obviously, the five treated strains showed faster growth speed than the control (Figure 2a). The biomass of mycelium pretreated at 50 s, for instance, reached about 1.03 g per100 ml of fermentation broth in 3 days, 56.1% higher than that of the control group (0.66 g) with the same culture time. Moreover, compared with the control group (20.61 ± 0.69 μg/g biomass), the strain pretreated for 30 s showed an increase of 22.9% in penicillin accumulation (25.33 ± 0.17 μg/g biomass, Figure 2b). However, there was no significant difference in the accumulation of penicillin between the other four treatment groups and the control group. Like the influence of discharge power discussed above, short pretreatment time led to the low-level radiation and energy, which has little effect on mycelium. On the contrary, long pretreatment time produced a high level of radiation and energy, which would inhibit the secretion of penicillin in mycelium [24].

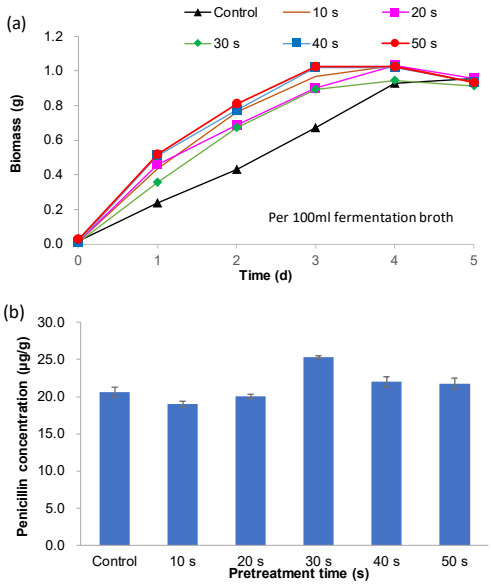


Figure 2: The effect of different pretreatment time of cold plasma on penicillin accumulation for P. chrysogenum. (a) mycelia growth; (b) penicillin concentration.

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Finally, the influence of cold plasma (70-150 Pa) with different helium working pressure on penicillin accumulation was measured under the condition that the discharge power and pretreatment time remained unchanged (120 W and 15 s). Results in Figure 3 show that the penicillin accumulation in the treatment group was the highest (18.64 ± 0.31 μg/g biomass) under the working pressure at 90 Pa. In comparison, that in the control group was only 15.03 ± 0.46 μg/g biomass. The moderate helium working pressure could produce moderate radiation and energy level, which was helpful to greatly improve the mycelium activity.

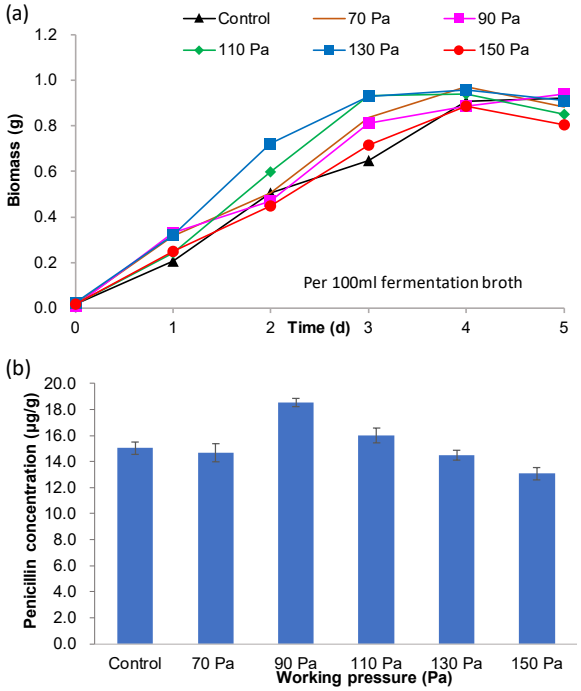

Figure 3: The effect of working pressure of cold plasma on penicillin accumulation for P. chrysogenum. (a) mycelia growth; (b) penicillin concentration.

3.2. The optimal parameters establishment for cold plasma pretreatment

On the basis of the single-factor test results discussed above, the orthogonal test (L9 (34), Table 1) was further conducted to optimize the pretreatment parameters for enriching penicillin.

Table 1: Factor and level values for orthogonal experiments

Factor	Coding	Level		
		1	2	3
Pretreatment time (s)	A	25	30	35
Discharge power (W)	B	130	140	150
Working pressure (Pa)	C	80	90	100

From the range (R), parameters of importance were discharge power (B), working pressure (C) and pretreatment time (A) in turn (Table 2). Through orthogonal test, under the optimized condition of 35 s, 130 W and 100 Pa (A3B1C3), the accumulation of penicillin reached the highest (34.95 μg/g biomass), which was 79.7% higher than that of the control.

To sum up, we guessed that effects of plasma pretreatment on microbial cells was probably related to the radiation and energy generated by plasma, just like the sun on human beings. Low radiation and energy were not enough to have effects on cells, while high one was destructive to them. In contrast, moderate radiation and energy could be absorbed by biological macromolecules in cells, such as nucleic acids and proteins, further producing some positive biological effects (e.g., enhanced metabolic activity of the cells).

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Table 2: The results of orthogonal experiments

No	A	B	C	Blank column	Penicillin concentration (μg/g biomass)
1	1	1	1	1	32.54
2	1	2	2	2	31.78
3	1	3	3	3	32.21
4	2	1	2	3	32.95
5	2	2	3	1	33.08
6	2	3	1	2	32.16
7	3	1	3	2	34.95
8	3	2	1	3	32.98
9	3	3	2	1	31.85
K1	32.18	33.48	32.56		
K2	32.73	32.61	32.19		
K3	33.26	32.07	33.41		
R	1.08	1.41	1.22		

4. Conclusion

This study successfully increased penicillin accumulation for P. chrysogenum pretreated with helium RF cold plasma. This work would develop an efficient and green approach for the production of industrial antibiotics and broaden the application field of cold plasma on the other secondary metabolites.

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