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Enhancing Extracellular Cordycepin Production In Engineered Pichia Pastoris Using Surfactants

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Abstract:
Cordycepin, chemically known as 3'-deoxyadenosine, is a biologically active natural compound with anticancer, antiviral, and immunomodulatory properties, etc. It holds great potential as a therapeutic agent and functional ingredient. With the identification of the cordycepin biosynthetic gene cluster in Cordyceps militaris, various yeast platforms have been engineered to establish cell factories for cordycepin synthesis, addressing the limitations of low yield and high cost associated with chemical synthesis and extraction from natural sources. Our laboratory previously developed a Pichia pastoris strain THP292 capable of producing cordycepin. In this study, we explored the use of surfactants to enhance extracellular cordycepin titers by improving membrane permeability. The effects of different concentrations of non-ionic surfactants, including Tween 60, Tween 80, and Triton X-100, on cell viability and cordycepin yield over time were investigated. The optimal surfactant type and concentration were identified, demonstrating that surfactants can significantly increase extracellular cordycepin production without negatively affecting cell growth. These findings provide a novel approach for improving cordycepin production processes.

Key Word*: Cordycepin; Surfactant; Pichia pastoris; Fermentation.*

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I. Introduction

Cordycepin, the first nucleoside antibiotic isolated from fungi, is structurally characterized as 3'-deoxyadenosine with the chemical formula $C_{10}H_{13}N_5O_3$[1]. It exhibits a wide range of biological activities[2-4], including anticancer, antiviral, immune modulation, and antimicrobial effects. Cordycepin inhibits the proliferation and metastasis of various tumor cells by regulating cytokine expression, such as IL-2, IL-4, TNF-α, TGF-β, and IFN-γ[5]. It also shows hypoglycemic activity in diabetic mice induced by alloxan and can reduce obesity in high-fat diet-induced rats by modulating the gut microbiota[6, 7]. Due to its versatile biological functions, cordycepin is widely regarded as a promising candidate for therapeutic applications in functional foods, health supplements, and cosmetics[8].

Currently, cordycepin is mainly extracted from *Cordyceps militaris*, which faces challenges such as long fungal culture cycles, low yield, and complex purification processes. Although chemical synthesis of cordycepin is possible through semi-synthetic and total synthetic routes, these methods suffer from low efficiency, high costs, and significant environmental impact[9, 10]. The completion of *C. militaris* genome sequencing has revealed the cordycepin biosynthetic pathway[11], including key gene clusters such as *Cns1-4*, which synthesize 3'-AMP as the core precursor. Similar gene clusters, *ck1-ck4*, were identified in *Cordyceps kyushuensis Kob* through transcriptomic and proteomic analyses. These discoveries have enabled the construction of heterologous cell factories for cordycepin biosynthesis in various microbial platforms.

In recent years, engineered microorganisms such as *Saccharomyces cerevisiae*, *Yarrowia lipolytica*[12], and *Aspergillus oryzae*[13] have been developed for the production of cordycepin. Our laboratory has constructed a *Pichia pastoris* strain, THP292[14], capable of synthesizing cordycepin; however, further enhancements in cordycepin yield are required through both genetic engineering and process optimization. While previous studies have focused on optimizing fermentation medium composition and culture conditions, the role of membrane permeability in enhancing extracellular cordycepin secretion remains underexplored. Surfactants[15], which reduce surface tension and alter membrane permeability, can potentially increase the extracellular release of cordycepin without affecting yeast cell viability. This study aims to investigate the effects of non-ionic surfactants—Tween 60, Tween 80, and Triton X-100—on cordycepin production and cell growth in *P. pastoris*, identifying the optimal surfactant and concentration to maximize extracellular cordycepin yield.

II. Material And Methods

Strains and Media

The *Pichia pastoris* GS115 strain and the engineered strain *P. pastoris* THP292[14] were used in this study. The media used were as follows:

YPD medium (100 mL): Glucose 2 g, peptone 0.5 g, yeast extract 0.5 g, supplemented with bleomycin with a final concentration at 100 μg/mL for strain activation.

BMG induction medium (1 L): Glycerol 10 mL, K_2HPO_4 3.01 g, KH_2PO_4 11.81 g, $(NH_4)_2SO_4$ 10 g, YNB (without ammonium sulfate) 3.4 g, and 0.2% (w/v) biotin 2 mL.

BMM synthesis medium (1 L): Methanol 0.5% (v/v), K_2HPO_4 3.01 g, KH_2PO_4 11.81 g, $(NH_4)_2SO_4$ 10 g, YNB without ammonium sulfate 3.4 g, and 0.2% (w/v) biotin 2 mL.

Fermentation Experiments

The seed culture of *P. pastoris* THP292 was inoculated into 50 mL YPD medium and incubated at 30°C, 180 rpm for 24 hours. Subsequently, 500 μL of the seed culture was transferred to 50 mL BMG medium with glycerol as the carbon source, supplemented with 100 μL histidine, and incubated at 30°C, 180 rpm for 24 hours. The cells were then centrifuged, washed with sterile water and BMM medium, and resuspended to a consistent starting OD_{600} across different experimental groups. The cultures were incubated at 30°C, 180 rpm, with 300 μL methanol added every 24 hours.

Surfactant Addition

Three non-ionic surfactants—Tween 60, Tween 80, and Triton X-100—were added to the cultures at the beginning of BMM medium induction, at concentrations of 1.0, 2.0, 4.0, 5.0, and 8.0 g/L. Each condition was tested in triplicate. Samples were collected at 48, 120, and 168 hours to measure cell biomass (OD_{600}) and cordycepin concentration in the supernatant using high-performance liquid chromatography (HPLC) as previously described[14].

III. Result

Our laboratory previously developed the engineered *P. pastoris* strain THP292 for cordycepin production, and we demonstrated that cordycepin can be secreted extracellularly. By modifying membrane permeability, the extracellular yield of cordycepin could be further increased. Here, we systematically evaluated the effects of the non-ionic surfactants Tween 60, Tween 80, and Triton X-100 on cordycepin production and cell growth, providing insights into process engineering optimization for large-scale fermentation.

Effect of Tween 60 on Cordycepin Production

Tween 60 was added at the start of fermentation, and both cell biomass (OD_{600}) and cordycepin concentration were measured at 48 h, 120 h, and 168 h. As shown in Figure 1, Tween 60 did not exhibit any inhibitory effect on the viability of *P. pastoris* THP292 cells. In fact, compared to the control, the OD_{600} values of the experimental groups with Tween 60 were slightly higher, indicating that Tween 60 promotes the growth of *P. pastoris* within the tested concentration range. Optimal growth was observed at a concentration of 2.0 g/L of Tween 60.

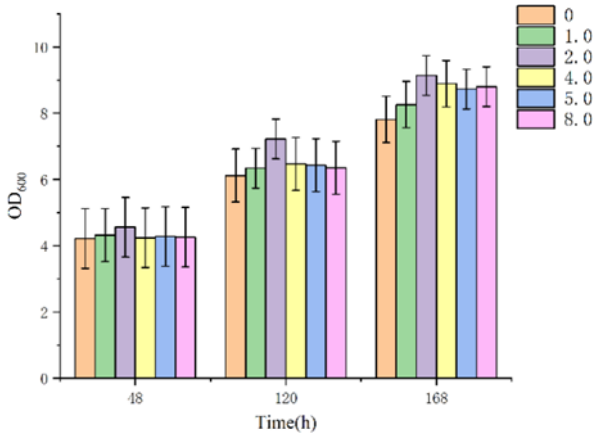

Figure 1. Effect of Tween 60 on the Growth of *Pichia pastoris*

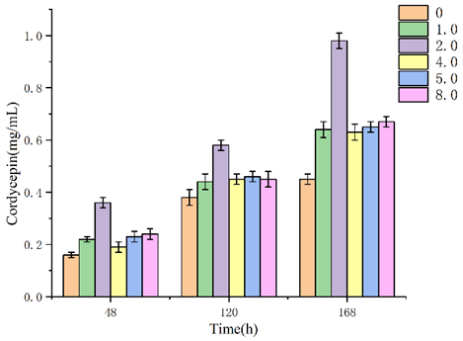

Figure 2. Effect of Tween 60 on Cordycepin Production

As shown in Figure 2, the cordycepin concentration in the fermentation broth was significantly higher in the group treated with 2.0 g/L Tween 60 than in other groups. At 168 h, the cordycepin yield in the 2.0 g/L Tween 60 group reached 2.14 times that of the control, demonstrating the substantial potential of Tween 60 in enhancing cordycepin production without negatively impacting cell growth.

Effect of Tween 80 on Cordycepin Production

Tween 80 was also added at the beginning of fermentation, and cell biomass (OD_{600}) and cordycepin concentrations were measured at 48 h, 120 h, and 168 h, as shown in Figures 3 and 4. Tween 80 did not inhibit the growth or fermentation of *P. pastoris*. In comparison to the control, the experimental groups treated with Tween 80 exhibited a slight increase in biomass, and extracellular cordycepin yields increased by 5% to 30%. Particularly, at a concentration of 4.0 g/L, cordycepin production increased by 30.8% compared to the control at 168 hours, with the highest biomass growth also observed at this concentration. However, at 5.0 g/L, cordycepin production showed a significant decline compared to 4.0 g/L, suggesting that 4.0 g/L is the optimal concentration. By comparing the data at different time points, it was observed that Tween 80 exhibited a significant effect as early as 48 hours, with the optimal concentration being 4.0 g/L. At this concentration, cordycepin production exceeded that of the control by more than twofold.

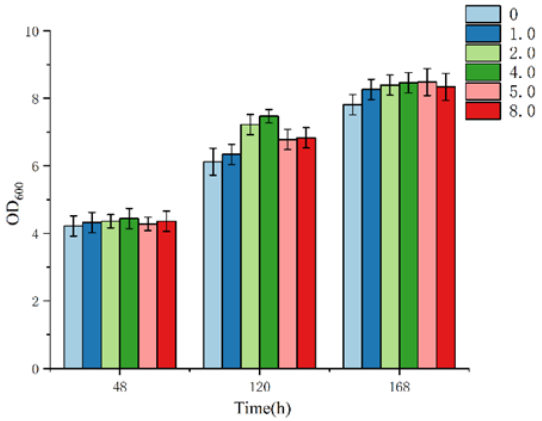

Figure 3. Effect of Tween 80 on the Growth of *Pichia pastoris*

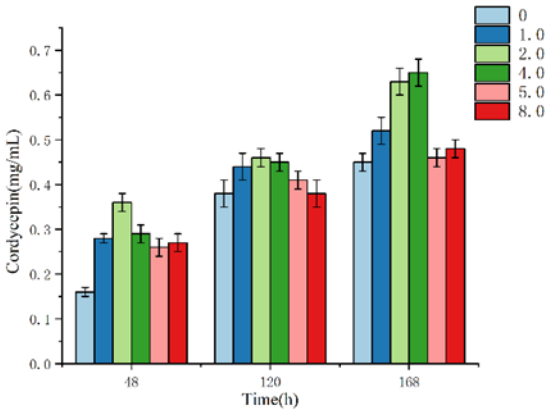

Figure 4. Effect of Tween 80 on Cordycepin Production

Effect of Triton X-100 on Cordycepin Production

Triton X-100 was added at the start of fermentation, and cell biomass (OD_{600}) and cordycepin concentrations were measured at 48 h, 120 h, and 168 h, as shown in Figures 5 and 6.

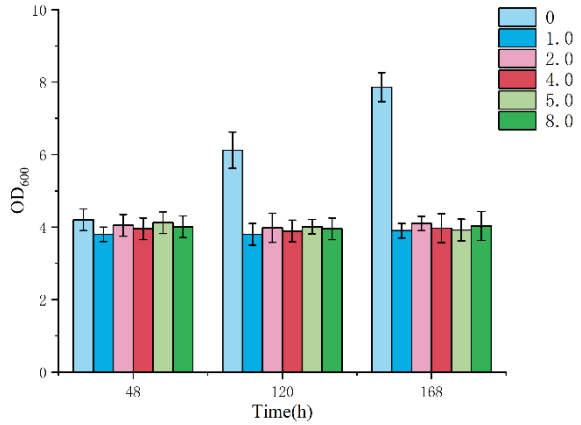

Figure 5. Effect of Triton X-100 on the Growth of *Pichia pastoris*

Figure 5 shows that even at low concentrations, Triton X-100 significantly inhibited the growth of *P. pastoris*, particularly after 120 h, with inhibition rates exceeding 40%. These findings indicate that Triton X-100 is not conducive to *P. pastoris* fermentation and is unsuitable as a surfactant for optimizing *P. pastoris* fermentation.

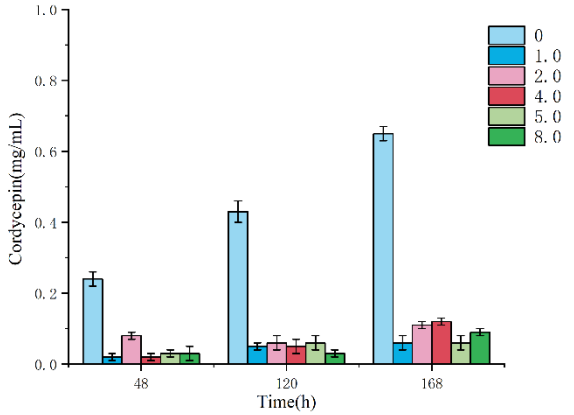

Figure 6. Effect of Triton X-100 on Cordycepin Production

As shown in Figure 6, cordycepin production in the fermentation broths with Triton X-100 was significantly reduced. As early as 48 h, inhibition rates of 60% to 94% were observed across all concentrations tested, further confirming that Triton X-100 is not suitable for optimizing *P. pastoris* fermentation for cordycepin production.

IV. Discussion

This study evaluated the effects of different surfactants, including Tween 60, Tween 80, and Triton X-100, on the growth of *P. pastoris* and the extracellular concentration of cordycepin, aiming to identify the optimal surfactant and its effective concentration.

The results revealed that both Tween 60 and Tween 80 enhanced the growth of *P. pastoris* and increased cordycepin production without exhibiting inhibitory effects. In contrast, Triton X-100 significantly inhibited both cell growth and cordycepin synthesis, indicating that it is not suitable as a surfactant for optimizing *P. pastoris* fermentation. Among the tested surfactants, Tween 60 demonstrated the best performance, with an optimal concentration of 2.0 g/L.

In recent years, various microbial platforms, such as *Y. lipolytica* and *S. cerevisiae*, have been used for the total synthesis of cordycepin. The application of surfactants can also be extended to other microbial hosts for cordycepin synthesis, offering a cost-effective and efficient process engineering strategy for large-scale production.

V. Conclusion

The addition of 2.0 g/L of the surfactant Tween 60 significantly enhances the extracellular production of cordycepin in *P. pastoris* and promotes cell growth. This makes Tween 60 an effective process engineering strategy for improving cordycepin yields in fermentation processes.

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