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Study On The Fermentation Method Of Astaxanthin Feed Produced By Solid-State Fermentation Of *Phaffia Rhodozyma*

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Abstract:
Background*:Astaxanthin is a fat-soluble carotenoid with strong antioxidant activity, which can effectively enhance animal immunity and has coloring function.*
Materials and Methods*: In this study, the fermentation method of solid-state fermentation of astaxanthin with Phaffia rhodozyma was evaluated. Two fermentation methods were studied, namely two-stage fermentation with aerobic culture and sealed culture, and reserved air fermentation. After two methods of fermentation, the astaxanthin content and acid soluble protein/crude protein relative content in the feed were selected as the main indicators for weighted evaluation.*
Results*:The results showed that in the two-stage fermentation experiment, aeration fermentation for two days was the best. In the reserved air test fermentation experiment, a 20% air reservation is optimal.*
Key Word*: Astaxanthin; Solid-state fermentation; Phaffia rhodozyma*

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I. Introduction

Astaxanthin is a kind of red carotenoid that can be used as a natural coloring agent and feed additive. Astaxanthin also has the antioxidant role of quenching singlet oxygen. Its antioxidant activity is approximately 10 times higher than that of other carotenoids (e.g., zeaxanthin, lutein, tunaxanthin, cantaxanthin, and β-carotene) and 100 times greater than vitamin E (α-tocopherol)(Miki, 1991; Ambati et al.,2014). It is used commercially in the food, animal feed, cosmetic, and pharmaceutical industries (Nutakor, et al.2022).

The biotechnological production of astaxanthin can be based on the utilization of waste from aquatic product processing, *Haemittococcus pluvialis*, or *Phaffia rhodozym*. *Phaffia rhodozym* is one of the most promising microorganisms for the commercial production of astaxanthin. Phaffia rhodozyma can produce astaxanthin at a relatively high growth rate and is a good source of proteins, lipids, and vitamins (Mussagy et al., 2021; Zhuang, 2021).

Solid-state fermentation is a simple and effective feed treatment method. Solid state fermented feed can not only improve feed utilization but also have beneficial effects on livestock and poultry. Solid fermentation feed can improve product quality and palatability, and protease secreted by microorganisms can decompose anti nutritional factors such as trypsin inhibitor, improve digestion and absorption rate and feed value, reduce production costs and environmental pollution. Moreover, solid-state fermented feed is widely used by feed fermentation enterprises due to low equipment investment and low production costs.

In this study, the method of solid-state fermentation using *Phaffia rhodozyma* to produce astaxanthin feed was studied. The content of astaxanthin and acid soluble protein/crude protein relative content in fermented feed can be increased by solid state fermentation.

II. Material And Methods

Medium

PDA Solid medium : potato extract powder 6g， glucose 20g，Agar powder 20g，and 1000 mL of water, with nature pH, sterilization at 121 ℃ for 20 minutes.

YM liquid medium: glucose 10 g, peptone 5 g, yeast extract 3 g, malt extract 3 g, water 1000 mL, pH 6.0-6.4, sterilization at 121 ℃ for 20 minutes.

The fermentation base consists of 60% corn meal, 25% soybean meal and 15% bran， sterilization at 121 ℃ for 30 minutes.

Strain Culture

*Phaffia rhodozyma P*4 was cultured on PDA slant medium at 22 ℃ for 72 hours. Then, inoculate the slant culture into YM liquid culture medium at 22 ℃, 200 r/min, for 72 hours.

Basic fermentation conditions

Inoculate the sterilized fermentation substrate, and the inoculation amount was 1.0×10^9 CFU/kg, adjust the moisture content to 40%, set 3 parallels for each processing, and then place it in a 250 mL triangular flask, at 22 ℃, fermentation for 8 days. Based on these, the effects of aeration fermentation time and air reservation on the main performance indicators of fermented feed were studied.

The influence of aeration fermentation time on the main indicators of fermented feed

After loading the fermentation material into the fermentation bottle, we adopt a two-stage fermentation method. In the first stage, the fermentation bottles were sealed the breathable membrane and incubated for 1 d, 2 d, 3 d and 4 d respectively. In the second stage, the fermentation bottles were sealed and continue to cultivated for 8 days. After 8 days of fermentation, take an appropriate amount of sample to measure pH, astaxanthin content, acid soluble protein/crude protein relative content. Using astaxanthin content accounting for 60% and acid soluble protein/crude protein relative content for 40%, a weighted scoring calculation was conducted to determine the optimal air reserve for fermented feed.

The influence of air reservation on the main indicators of fermented feed

Fill the fermentation material into the fermentation bottle with a mass of 100%, 90%, 80%, and 70% of the full mass, which means that the air reserve is 0%, 10%, 20%, and 30%, respectively. After 8 days of fermentation, We measured the samples. The determination method for fermented feed samples was the same as the previous section.

Data Analysis

All indicators were measured to ensure that there were three duplicate samples. The experimental data were statistically analyzed using Excel 2019 and SPSS software, and one-way analysis of variance (ANOVA) was used. Multiple comparisons between groups were conducted using Duncan's analysis for significance of differences, and the results were expressed as mean ± standard deviation.

III. Result

The influence of aeration fermentation time on the main indicators of fermented feed

The main indicators of fermented feed under different aerobic times were shown in Table 1. From Table 1, it can be seen that the pH of fermented feed with different aerobic fermentation times was significantly lower than that of fermentation substrate ($P<0.01$). Among them, the pH of fermented feed with aerobic fermentation for 2 days can be reduced to 4.26.

Based on the weighted scores of astaxanthin content and acid soluble protein/crude protein relative content, it can be seen that the highest score was the aerobic fermentation time of 2 days. Therefore, the optimal aerobic fermentation time was 2 days.

Table 1 The influence of aeration fermentation time on the main indicators of fermented feed

Aeration fermentation time(d)	pH	Astaxanthin content （mg/kg）	Acid-soluble protein/crude protein (%)	Weighted score
0	4.50 ± 0.16^{bcBC}	17.21 ± 0.23^{cB}	26.27 ± 1.40^{abA}	20.84
1	4.32 ± 0.16^{cC}	18.50 ± 0.22^{bA}	26.67 ± 0.78^{abA}	21.77
2	4.26 ± 0.11^{cC}	19.38 ± 0.61^{aA}	27.19 ± 1.19^{aA}	22.50
3	4.37 ± 0.08^{cC}	18.60 ± 0.43^{bA}	26.07 ± 1.24^{abA}	21.56
4	4.75 ± 0.13^{bB}	17.41 ± 0.61^{cB}	24.73 ± 0.86^{bA}	20.34
Fermentation substrate	6.49 ± 0.17^{aA}	0^{dC}	14.18 ± 0.23^{cB}	5.67

Note: Different lowercase letters on the shoulder mark in the same column show significant difference ($P<0.05$), and different uppercase letters on the shoulder mark show extremely significant difference ($P<0.01$), the same below.

The influence of air reservation on the main indicators of fermented feed

The main indicators of fermented feed under different air reserves were shown in Table 2. From Table 2, it can be seen that the pH of fermented feed with different air reserves was significantly lower than that of fermentation substrate ($P<0.01$). Under 10% air reserve, the pH of fermented feed was 4.33. Under 30% air reserve, the astaxanthin content in fermented feed was the highest, reaching 18.12%. Under 10% air reserve, the acid soluble protein/crude protein relative content in fermented feed was the highest, reaching 27.12%.

Based on the weighted scores of astaxanthin content and acid soluble protein/crude protein relative content, it can be seen that the highest score was the air reservation of 20%.

Table 2 The influence of air reservation on the main indicators of fermented feed

Air reserve	pH	Astaxanthin content （mg/kg）	Acid-soluble protein/crude protein (%)	Weighted score
0%	4.36 ± 0.11^{cB}	17.17 ± 0.56^{bA}	26.73 ± 0.84^{aAB}	21.00
10%	4.33 ± 0.16^{cB}	17.62 ± 0.44^{abA}	27.12 ± 0.50^{aA}	21.42
20%	4.51 ± 0.16^{bcB}	17.90 ± 0.57^{abA}	26.80 ± 0.68^{aAB}	21.46
30%	4.63 ± 0.14^{bB}	18.12 ± 0.38^{aA}	24.56 ± 1.39^{bB}	20.70
Fermentation substrate	6.60 ± 0.08^{aA}	0^{cB}	14.01 ± 0.60^{cC}	5.60

IV. Discussion

The use of *Phaffia rhodozyma* for solid-state fermentation of feed can significantly reduce the pH of the fermentation substrate and increase the relative content of acid soluble protein/crude protein. The digestive enzyme produced by *Phaffia rhodozyma* during its growth can degrade protein, polysaccharide and other macromolecular substances, and then produce a variety of organic acids after further metabolism, thus reducing the pH. Degradation products such as peptides, small peptides, and amino acids can increase the relative content of acid soluble protein/crude protein(Su et al., 2018; Sun et al.,2012). At the same time, astaxanthin can be directly produced in feed, thereby improving feed quality and increasing nutritional value(Conradie et al., 2018; Zhu et al.,2022).

The two-stage method was used for feed solid-state fermentation. In the first stage, when it is in the aerobic fermentation state, *Phaffia rhodozyma* can grow and proliferate rapidly, providing a sufficient number of living cells for the subsequent anaerobic fermentation process, while producing a variety of digestive enzymes, providing a basis for the full decomposition and utilization of the substrate.

Another fermentation method of reserving air can also enable rapid growth and proliferation of *Phaffia rhodozyma*. Due to the limited oxygen content of the reserved air, the weighted score value is slightly lower compared to the two-stage fermentation method. That is, the two-stage fermentation method corresponds to higher astaxanthin content and acid soluble protein/crude protein relative content, resulting in better fermentation effect. However, in large-scale production, considering factors such as ease of operation, reserved air can be chosen to meet the aerobic fermentation process of *Phaffia rhodozyma*. The weighted scoring values of 10% and 20% reserved air are not significantly different. Therefore, considering production costs and other factors, a 10% reserved air can be selected for subsequent solid-state fermentation research.

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