Study on optimum technological conditions for producing androstenedione by microbial method

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Abstract. As an indispensable intermediate, androstenedione is widely used in drug manufacturing, especially steroidal drugs. However, the chemical manufacturing process of androstenedione is generally complicated and difficult, and it will cause serious environmental pollution in the production process. The biological method for the production of androstenedione has a very promising development prospect, because it is more economical and environmentally friendly than chemical methods. In order to better produce androstenedione on a large scale, the imbalance between supply and demand can be solved. In this study, the biphase system was used to increase the substrate concentration, and the method of transforming plant sterol by mycobacterium was used to produce androstenedione. The optimal conditions for the production of androstenedione by microbial assay were determined by orthogonal test: the aqueous two-phase system was water/ sunflower oil, the temperature was 30 °C, the initial pH was 6.5, the substrate concentration was 0.4 g/L, the rotation speed was 250 rpm, and the inoculation was carried out. The amount was 14.83%, the organic ratio was 20.65%, and the liquid loading was 150/500 mL. The preliminary production of androstenedione by microbial method has found suitable process conditions and provided data and theoretical support for its large-scale production.

1 Introduction

Anthraquinone is a widely used drug. According to statistics, there are more than 150 steroids currently used for clinical treatment ^[1]. Terpenoids generally contain a steroidal nucleus, which is the reason for its special structure^[2]. In recent years, due to the continuous development of medicine, the demand for steroids has surged^[3], natural extraction methods have been unable to meet market demand, and artificial synthesis has become new access to steroids. Androstenedione (AD) is an essential intermediate for the synthesis of most steroids in this context ^[4]. It is produced by the traditional method of extracting diosgenin from the resources of the genus *dioscorea* ^[5] and then further synthesizing it by chemical methods.

Fig.1 Structure of androstenedione

However, this method of natural extraction plus chemical synthesis requires harsh reaction conditions and generally requires the use of a special catalyst ^[6]. And the steps are numerous, the synthesis is difficult, the unit price is even higher up to 1000 yuan/kg ^[7].

It is subject to other natural factors such as weather, seasons and geography because of the growth cycle of natural raw materials ^[8]. A variety of harmful substances such as organic strong acids and heavy metals are present in the waste liquid produced in the process of synthesizing AD by this method ^[9], it is not only difficult to deal with the waste liquid, but also causes unrecoverable pollution to the water environment and soil environment ^[10].

With the continuous development of biological downstream technology, microbial transformation has become a new way to obtain AD. AD with good dispersibility and stability can be produced by the degradation of phytosterols ^[11] by many bacteria, such as *Nocardia*, *Arthrobacter* and *Mycobacterium* ^[12]. The microbial transformation method has the advantages of simple steps, low cost, mild reaction conditions, and less subject factors, compared with the traditional natural extraction and chemical synthesis methods. Moreover, the remaining fermentation broth still has the value of being reused after the AD in the culture solution is extracted ^[13]. The recovered oil meets the national secondary oil standard after refining, and can be used as a raw material

for biodiesel or recycled in the factory. In addition, it is also possible to recover a highly purified protein that can be recycled from the mash of the extracted oil by leaching.

Phytosterols are cheap and easy to obtain as a substrate, but it is difficult to dissolve in water [14]. In order to bring the mycobacteria into full contact with the substrate, an oil/water two-phase system was constructed to increase the substrate concentration [15], thereby increasing the yield. In addition to the type of oil phase, fermentation temperature [16], initial pH, and substrate concentration are all factors that affect AD yield. The purpose of this study was to find out the optimal conditions for the production of AD by microbial transformation, and to provide a technical reference for the large-scale production of AD plants.

2 Materials and methods

2.1 Materials

2.1.1 Bacterial source

Mycobacterium sp. BD-696, College of Food and Biological Engineering, Hefei University of Technology.

2.1.2 Reagents and instruments

Main reagents: Phytosterol, Sinopharm Chemical Reagent Co., Ltd. Androstenedione, Sinopharm Chemical Reagent Co., Ltd. Ethyl acetate, Shanghai Maclean Biochemical Technology Co., Ltd. Methanol, Sinopharm Chemical Reagent Co., Ltd. And n-hexane, Shanghai Zhongqin Chemical Reagent Co., Ltd.

Main instruments: vertical pressure steam sterilizer yxq-100sii, medical equipment factory of Shanghai boxun industrial Co., Ltd. Bs-1e constant temperature oscillating table, jintan chengxi fuwei experimental instrument factory. Medical refrigerator hyc-260, Qingdao haier special electric appliance Co., Ltd. Tgl-15b, high-speed table centrifuge, Shanghai 1.2.2 Solid Culture Method

Culture medium formula: beef extract 0.3%, peptone 1.0%, NaCl 0.5%, agar 1.5-2.0%; pH 7.0-7.2.

Cultural method: the strains were purified by plate scribing and dilution to obtain a single colony, and cultured at 30 ° C in a constant temperature incubator for observation and reserve [19].

2.1.3 Seed Culture Method

Culture medium formula: beef extract 0.68%, molasses 5.4%, glucose 0.6%, potassium dihydrogen phosphate 0.1%, GPE 0.007%, pH 8.3-8.5 [20].

Cultural method: the purified single colonies were picked from the solid medium using an inoculating loop, inoculated into a liquid medium, and cultured in a constant temperature oscillating shaking table at 30°C and 120 r/min for 3 days.

2.1.4 Fermentation culture method

Culture medium formula: beef extract 0.68%, oil 15%, molasses 5.4%, glucose 0.6%, potassium dihydrogen phosphate 0.1%, GPE 0.007%. Temperature, pH, and substrate (phytosterol) concentration were variables.

Cultural method: A certain volume of the bacterial liquid was aspirated from the liquid seed culture solution in the fermentation broth, and fermented for 7 days in a constant temperature shaker at 160 r/min [21].

2.1.5 Research methods for strain growth

Samples of the bacterial solution taken every 6 hours during the seed culture process, diluted 10⁵ times with pure water, and use an ultraviolet spectrophotometer to measure the absorbance of the diluted bacterial anting scientific instrument factory. Agilent high performance liquid chromatograph 1-3000, Beijing puyuan jingdian technology Co., Ltd. Dhg-9030a, Shanghai sanfa scientific instrument Co., Ltd. Shp-250fe, Shanghai sanfa scientific instrument Co., Ltd. Uv-spectrophotometer evolution300/600, Thermo Fisher Scientific-Shanghai.

2.2 Methods

2.2.1 Orthogonal experiment method

Using the orthogonal test method [17], the pH and temperature of the aqueous two-phase fermentation system, the concentration of the substrate (phytosterol), and the type of vegetable oil are used as variables. The correspondence between the types of organic phases and numbers is shown in Table 1. And the orthogonal test was designed by SPSS and its results are shown in Table 3.

After the optimal pH, temperature and substrate concentration are determined [17], the four factors of shaking speed (A), inoculum (B), organic phase (C) and liquid loading (D) were selected for response surface analysis [18] to more comprehensively determine the best microbial production of AD. Process conditions. The values of each factor are shown in Table 2. Based on the Design principle of Box-Behnken combination design, 29 test points were designed by Design-Expert 8.0 software. The response surface design and data results are shown in Table 7.

Table 1. Organic species

Number	1	2	3	4	5
Vegetable oil kind	Sunflower oil	Soybean oil	Rapeseed oil	Methanol	Polyethylene glycol

2.2.2 Solid Culture Method

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Cultural method: A certain volume of the bacterial liquid was aspirated from the liquid seed culture solution in the fermentation broth, and fermented for 7 days in a constant temperature shaker at 160 r/min ^[21].

Table 2. Design of response surface factors and levels

Level	Factor			
	A (Speed) /rpm	B (Inoculum size) /%	C (The ratio of the organic phase) /%	D (Loaded liquid) / (mL·500mL ⁻¹)
-1	150	5	10	50
0	200	15	20	100
1	250	25	30	150

2.2.7 Yield calculation method

AD standard solution with AD concentrations of 0.0001 g/L, 0.001 g/L, 0.01 g/L, 0.1 g/L and 1 g/L with ethyl acetate as solvent. HPLC was performed with the above method. The standard curve regression equation for peak area of concentration and spectrum of AD was established to calculate the concentration of AD under various conditions ^[24].

The calculation formula of yield is as follows:

$$Yield = \frac{m_p}{m_s} \times \frac{M_s}{M_p} \times 100\% \quad (1)$$

3 Result and analysis

3.1 Strain growth curve

The growth curve of the strain is shown in Figure 2.

2.2.5 Research methods for strain growth

Samples of the bacterial solution taken every 6 hours during the seed culture process, diluted 10⁵ times with pure water, and use an ultraviolet spectrophotometer to measure the absorbance of the diluted bacterial solution at a wavelength of 650 nm. The growth curve of the strain was plotted with the time coordinate as the abscissa and the absorbance as the ordinate to represent the growth of *Mycobacteria* BD-696.

2.2.6 Detection method

The supernatant of fermentation medium was taken, and the same volume of ethyl acetate was added to extract the produced AD, then ethyl acetate was used as solvent to dilute it 100 times to obtain the liquid to be tested. The content of AD in the samples was measured by the high performance liquid chromatograph (HPLC) ^[22]. The optimum condition was as follows: C18 column, mobile phase was methanol: water = 70:30, flow rate was 1 mL/min, column temperature was 30 °C, wavelength was 245 nm ^[23].

Fig. 2 Strain growth curve

Figure 2 shows that in the process of liquid culture, mycobacteria has an adaptation process when it is just inoculated into liquid medium, and some strains are difficult to adapt or even die, which is the reason why the dry weight of the cells will decrease. After about 20 h, the strain enters the logarithmic growth phase after the lag period has elapsed. At about 70 h, the dry weight of the strain peaked and entered a stationary phase. Therefore, it is preferred to inoculate the strain from the liquid medium to the fermentation medium at 70 h.

3.2 Liquid chromatography standard curve

The standard curve for the detection of AD by liquid chromatography is shown in Figure 3:

Fig. 3 HPLC curves of 0.01g/L androstenedione

It can be seen from Fig. 3 that under this liquid chromatography condition, the peak time of AD is about 4.8 min.

Fig 4 Liquid phase standard curve of androstenedione

Through the linear fitting in figure 4, the standard curve regression equation of the peak area calculated by the concentration of AD and the spectral diagram is as follows:

$$y = 4.3092 \times 10^{-5} x - 0.00212 \quad (2)$$

Where y is the AD concentration after the sample is diluted 100 times, and x is the peak area of the liquid chromatography.

3.3 Orthogonal test results

The results of orthogonal experiment are shown in table 3

Table 3. Orthogonal test results

Number	Temperature/ (°C)	pH	Substrate concentration/ (g/L)	Vegetable oil kind	AD concentration / (g/L)	Yield/%
1	30	8.5	0.8	3	3.77	64.26
2	40	7.5	0.8	5	1.21	20.71
3	35	8.0	0.8	4	3.63	61.95
4	20	6.5	1.0	4	3.63	49.58
5	20	8.0	0.8	2	3.77	64.30
6	40	9.0	0.6	4	1.22	27.80
7	40	6.5	0.4	1	1.97	67.33
8	40	8.5	0.4	2	1.19	40.55
9	30	7.0	0.8	4	4.62	78.70
10	20	8.0	0.4	3	2.43	82.88
11	30	7.5	0.4	1	2.34	79.75
12	30	6.0	0.4	2	2.47	84.29
13	35	7.5	1.0	2	1.29	17.54
14	25	7.0	0.8	2	4.56	77.82
15	30	9.0	0.8	5	4.28	72.99
16	35	8.5	0.8	1	4.20	71.59
17	25	6.5	0.8	2	4.28	72.97
18	20	7.0	0.8	1	4.44	75.71
19	40	7.0	1.0	3	4.35	59.35
20	35	9.0	0.4	1	2.26	76.94
21	20	9.0	0.8	2	3.80	64.85
22	40	8.0	0.8	2	0.74	12.69
23	20	8.5	0.4	4	1.29	44.13
24	30	6.5	0.6	2	3.27	74.39
25	25	6.0	0.8	4	4.42	75.36
26	20	7.0	0.4	5	2.16	73.74
27	25	9.0	0.4	3	2.36	80.44
28	20	6.5	0.8	3	3.88	66.10
29	35	6.0	0.6	3	1.07	24.24
30	25	7.0	0.6	1	0.89	20.34
31	25	8.5	0.8	1	4.38	74.66
32	20	6.0	0.8	1	4.43	75.62
33	20	7.5	0.8	1	4.22	72.04
34	25	7.5	0.4	4	1.96	66.96

35	25	6.5	0.4	1	2.37	80.90
36	20	6.0	0.4	2	2.44	83.24
37	25	8.5	1.0	2	4.22	57.58
38	25	8.0	0.6	1	3.61	82.02
39	20	7.5	0.6	2	3.26	74.10
40	20	9.0	1.0	1	1.23	16.76
41	25	6.0	1.0	5	1.35	18.43
42	35	7.0	0.4	2	1.32	45.16
43	25	7.5	0.8	3	3.28	55.94
44	25	9.0	0.8	2	3.84	65.54
45	20	8.5	0.6	5	2.07	47.16
46	25	8.0	0.4	5	1.16	39.55
47	35	6.5	0.8	5	4.23	72.17
48	40	6.0	0.8	1	4.31	73.56
49	30	8.0	1.0	1	4.38	59.77

From the yield results in Table 3, the fermentation conditions with the highest yield were: temperature 30 °C, initial pH 6.0, plant sterol concentration 0.4 g/L, and water/soybean oil aqueous two-phase system under these conditions. The yield can be as high as 84.29%; the fermentation power curve is shown in Figure 5:

Fig 5 Concentration change chart under the highest yield fermentation conditions

As can be seen from Fig. 5, the rate of change of AD concentration is already low at about 144 h after the start of fermentation, so it is reliable to measure the fermentation conditions by using the AD yield at this time as the final result.

According to the above orthogonal test result table, relevant data analysis is carried out^[25], and the analysis results are shown in Table 4, Table 5 and Table 6:

Table 4. Normal test results

Item	Statistic	DOF	P
Kolmogorov-Smirnov	0.189	49	0.064
Shapiro-Wilk	0.856	49	0.102

Table 5. Mean response of factors

Level	Temperature	pH	Substrate concentration	Vegetable oil kind
1	63.59	62.32	67.67	66.21
2	62.04	69.06	50.01	59.75
3	73.66	61.54	65.22	61.89
4	52.80	55.29	39.86	57.78
5	43.14	57.59		49.25
6		57.13		
7		57.9		
Delta	30.52	13.77	27.81	16.96
Row rank	1	4	2	3

Table 6. Analysis of variance of factors

Variable	DEVSQ	DOF	MS	F	P
Temperature	3896.2	4	974.1	2.84	0.041
pH	844.7	6	140.8	0.41	0.868
Substrate concentration	4932.6	3	1558.1	4.52	0.010
Vegetable oil kind	1408.8	4	352.2	1.02	0.411

As can be seen from Table 4, the significant level $P=0.064>0.05$, and the distribution of the yield is close to the normal distribution, with statistical significance. As

can be seen from Table 5, the effect of temperature on the final yield is the greatest among the four different influencing factors, the substrate concentration is second,

the vegetable oil species is again, and the initial pH has the least effect on the final yield. This may be because *mycobacteria* *BD-696* is sensitive to temperature conditions, and slightly higher temperatures may reduce or even inactivate its activity. But the pH is not static throughout the fermentation process, the strain can adapt to changes in pH conditions during this process.

Comparing the mean response of each factor with respect to yield, the fermentation conditions with the highest AD yield were estimated to be 30 ° C, initial pH 6.5, plant sterol concentration 0.4 g/L, and water/sweet oil dual aqueous phase system.

The analysis results show that the concentration of

sterol as a substrate is not positively related to the yield, conversely, the yield is rather low when the substrate concentration is high. This indicates that mycobacteria cannot fully utilize and decompose all plant sterols when the substrate is at a high concentration. The unutilized plant sterols inhibited biochemical reactions which is mycobacteria produce AD.

3.4 Response surface test results

The response surface test results and correlation analysis are shown in the following table:

Table 7. Response surface test results

Number	A (Speed) / rpm	B (Inoculum size) / %	C (The ratio of the organic phase) /%	D (Loaded liquid) / (mL•500mL ⁻¹)	Yield/ (%)
1	200	15	30	150	69.13%
2	150	15	30	100	39.00%
3	200	15	20	100	66.86%
4	200	5	10	100	48.74%
5	200	25	10	100	51.38%
6	150	15	10	100	37.27%
7	150	5	20	100	22.99%
8	200	15	30	50	64.34%
9	250	15	20	50	84.23%
10	200	15	20	100	73.25%
11	250	25	20	100	68.79%
12	200	5	20	150	50.76%
13	250	15	10	100	82.62%
14	150	15	20	50	45.05%
15	200	25	30	100	53.19%
16	200	15	10	50	59.60%
17	250	15	30	100	83.43%
18	200	25	20	150	55.50%
19	200	5	30	100	49.11%
20	200	25	20	50	56.25%
21	200	15	20	100	69.13%
22	200	15	20	100	68.60%
23	150	25	20	100	25.72%
24	250	15	20	150	85.77%
25	200	15	10	150	65.55%
26	200	5	20	50	50.35%
27	250	5	20	100	72.70%
28	200	15	20	100	70.73%
29	150	15	20	150	48.00%

Table 8. Response surface test anova table

Sources of variance	SS	DOF	MS	F	P
Model	0.75	14	0.054	64.79	< 0.0001
A	0.56	1	0.56	679.25	< 0.0001

B	2.179E-003	1	2.179E-003	2.64	0.1267
C	1.420E-003	1	1.420E-003	1.72	0.2110
D	1.852E-003	1	1.852E-003	2.24	0.1566
AB	1.102E-003	1	1.102E-003	1.33	0.2674
AC	2.147E-005	1	2.147E-005	0.026	0.8742
AD	4.919E-005	1	4.919E-005	0.060	0.8108
BC	5.208E-005	1	5.208E-005	0.063	0.8054
BD	3.298E-005	1	3.298E-005	0.040	0.8445
CD	3.388E-005	1	3.388E-005	0.041	0.8425
A ²	0.016	1	0.016	19.40	0.0006
B ²	0.17	1	0.17	206.80	< 0.0001
C ²	0.010	1	0.010	12.55	0.0033
D ²	6.819E-006	1	6.819E-006	8.253E-003	0.9289
Residual	0.012	14	8.263E-004		
Lack of fit	9.246E-003	10	9.246E-004	1.59	0.3465
Pure error	2.322E-003	4	5.806E-004		
Overall error	0.76	28			

According to the results of the response surface test and the variance analysis, the regression model $P < 0.0001$, the model is significant; the missing term $P = 0.3465$, which is not significant. The coefficient of determination is 0.9848, which indicates that the model has a high degree of fitting. In addition, the primary term A, the secondary terms A², B², and C² have a significant effect on the yield ($P < 0.0001$). B, C and D had no significant effect on the yield of AD produced by Mycobacterium BD-696 within the range of parameters obtained by the experiment ($P > 0.05$). Through optimization analysis, the quadratic multiple regression model equation calculated by actual factors is:

$$\begin{aligned}
 Y = & -1.71498 + 0.013010A + 0.056532B + \\
 & 0.018046C + 8.13552 \times 10^{-4}D - 3.32001 \times 10^{-5}AB - \\
 & 4.63352 \times 10^{-6}AC - 1.40275 \times 10^{-6}AD + \\
 & 3.60827 \times 10^{-5}BC - 5.74247 \times 10^{-6}BD - \\
 & 5.82042 \times 10^{-6}CD - 1.98853 \times 10^{-5}A^2 - \\
 & 1.62305 \times 10^{-3}B^2 - 3.99757 \times 10^{-4}C^2 - \\
 & 4.10133 \times 10^{-7}D^2
 \end{aligned}
 \quad (3)$$

The contour map and response surface 3D map are shown in Figure 6-11.

a Contour plot of AB

b Surface of response for AB

Fig. 6. Interaction diagram of rotation speed and inoculation amount on AD yield

a Contour plot of AC

b Surface of response for AC

Fig. 7. Interaction diagram of rotation speed and organic ratio on AD yield

b Surface of response for BC

Fig. 9. Interaction diagram of rotation speed and loading volume on AD yield

a Contour plot of AD

a Contour plot of BD

b Surface of response for AD

Fig. 8. Contour and interaction diagrams of response surface analysis

b Surface of response for BD

Fig. 10. Interaction diagram of inoculation volume and loading volume on AD yield

a Contour plot of BC

a Contour plot of CD

Fig. 11. Interaction diagram of organic phase ratio and loading volume on AD yield

As can be seen from the figure, the higher the rotational speed, the higher the yield of AD. At higher rotational speeds, the microorganisms and the substrate are in full contact, and the product can be evenly distributed to the entire fermentation environment in time. Presumably that's why it doesn't affect the forward direction of the reaction. In the case of a higher inoculum, the yield of AD did not increase as it increased. Probably because when the inoculum is too large, the adequate growth of mycobacteria is hampered by insufficient nutrients, this affects its biological function of using the substrate to produce AD.

Less inoculation resulted in insufficient activity of the whole fermentation system, and of course, the yield of AD was low.

On the other hand, the yield can reach a higher value when the ratio of the organic phase to the liquid loading is moderate. The order of influence of each variable on the yield of AD is $A > B > D > C$. The yield of AD has a maximum at a relatively suitable rotational speed and inoculum size, and the rotational speed is higher at this time, while the inoculum amount, liquid volume, and organic ratio are moderate.

After optimization of the response surface, the optimal process conditions for the production of AD by the obtained microbial method are A (rotation speed) = 250 rpm, B (inoculation amount) = 14.83%, C (organic ratio) = 20.65%, D (loading amount) = 150/500 mL. The yield of AD under this optimal fermentation condition was predicted to be 87.18%.

When the optimization parameters are corrected in the results of the verification response surface analysis, they are: A (rotation speed) = 250 rpm, B (inoculation amount) = 15%, C (organic ratio) = 21%, D (loading amount) = 150/500 mL. Under these conditions, three trials were carried out to verify that the AD yields were 86.23%, 85.84%, and 84.96%, respectively, and the relative errors were 1.09%, 1.54%, and 2.55%, respectively, compared with the predicted values. Therefore, the model can predict the effect of rotational speed, inoculum size, organic phase ratio and liquid loading on AD yield under this fermentation condition.

4 Conclusions

The highest yield fermentation conditions in orthogonal

experiments were: temperature 30 °C, initial pH 6.0, plant sterol concentration 0.4 g/L, and water/soybean oil aqueous two-phase system. The yield under this condition can be as high as 84.29%.

The order of influence of different environmental influence factors on the final yield is: temperature > substrate concentration > vegetable oil type > initial pH. The optimal conditions for the production of AD by mycobacteria BD-696 in the two-phase system are: temperature 30 °C, initial pH 6.5, plant sterol concentration 0.4 g/L, water/sweet oil dual aqueous phase system.

On the basis of the orthogonal experimental results, the fermentation conditions with the highest yield in the actual experiment of response surface were: rotation speed 250 rpm, inoculum volume 15%, organic comparison example 20%, liquid loading 150/500 mL. The yield under this condition can be as high as 85.77%. The order of influence of each variable on the yield of AD was speed > inoculum > liquid volume > organic phase. The optimal fermentation conditions for the effective prediction of the response surface model are: speed of 250 rpm, inoculum size of 14.83%, organic ratio of 20.65%, and liquid loading of 150/500 mL. At this time, the yield of AD can be as high as 87.18%.

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