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The optimization of the production conditions of gamma aminobutyric acid by *Absidia* fermentation.

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Abstract

Gamma Aminobutyric Acid (GABA), also known as 4-aminobutyric acid, of which the molecular formula is $C_4H_9NO_2$, with a relative molecular weight of 103.2. It widely exists in the cells of microorganism, animals and plants, as a kind of neuro-inhibitory transmitter in mammals, a variety of physiological and metabolic activities are involved. It is greatly demanded by the market for the special physiological function and medicinal value with its unique molecular structure: it has the function of lowering blood pressure, treating epilepsy, enhancing memory, anti-sleeping and delaying senescence, with higher physiological activity. We used *Absidia* as the fermentation strain, and found out the best fermentation conditions: temperature: 31°C; time: 70 h; pH value: 4.9; inoculation age: 18 h, inoculation amount: 8%, rotation speed: 170 r/min. When the fermentation test was performed according to the conditions, the molar conversion rate of GABA can reach 32.03%, this can provide theoretical support for large-scale industrial production in the future.

Keywords: Gamma aminobutyric acid, Glutamic acid decarboxylase, Fermentation, Molar conversion rate.

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Introduction

In this experiment, Glutamic Acid Decarboxylase (GAD) was used to catalyse the removal of carboxyl group of L-Glutamic acid (L-Glu) to produce GABA [1-3], as GAD is the only enzyme that catalyses the L-Glu decarboxylation reaction to generate GABA [4], there's a huge difference of glutamic acid decarboxylase's distribution in different microbes [5], glutamic acid decarboxylase in bacteria mainly exist in the cytoplasm and mitochondria [6], in fungi mainly exist in the conidium, less content in substrate mycelium and aerial mycelium [7], however, the existence of glutamic acid decarboxylase in mycelium of *Absidia* provides a theoretical basis for the production of GABA by mycelium fermentation [8]. Besides, as a kind of mold, *Absidia* has the following advantages: easy cultivation, vigorous growth, rapid reproduction, strong anti-bacterial ability and strong ability to produce Glutamic Acid Decarboxylase (GAD) [9] , So we chose *Absidia* as the fermentative strain, the production of GABA by fermentation usually had the advantages of high utilization rate of glutamic acid, simple process flow, easy purification and short cycle, the optimization of fermentation conditions that make GAD achieve maximum effectiveness is essential to develop the yield of GABA [10]. The dry weight of bacteria can reflect its growth degree, generally, the higher the dry weight of bacteria is, the higher the yield and molar conversion rate of GABA is. Therefore, the best fermentation condition was determined by the dry weight and GABA molar conversion rate as reference index [11]. The previous production methods of gamma aminobutyric acid by *Absidia* fermentation mainly include the following defects: the optimal fermentation conditions are difficult to determine [12], and the yield is difficult to meet the requirements of mass production [13]. So the main purpose of this experiment is as follows: the fermentation conditions of GABA produced by *Absidia* should be optimized to achieve the highest molar conversion rate, in order to provide theoretical support for large-scale industrial production.

Methods

Culture of fungal strains

PDA: Culture of strains: the *Absidia* named MQ preserved by our lab, was cultured to be activated in the medium of Potato Dextrose Agar (PDA): 30°C, for 3 d.

Seed culture: The separated and activated mycelium of MQ strains was washed by sterile saline, 4 times. The mycelium was filter out with sterile cotton, and the filtrate was removed into the sterilized triangle flask. The fungal spore suspension was prepared by shaking the filtrate, adjusted to the spore concentration of 10^8 spores/L by hemacytometry, and eventually transferred to the seed medium (the mass fraction: 20% seed potatoes, 2% glucose, 2% agar, 76% distilled water, pH=6.5, sterilization: 121°C, 20 min), the liquid volume was 40 ml/250 ml, 31°C, 170 r/min, shaking cultured for 13 h.

Fermentation culture: The cultivated seed liquid was added into the fermentation medium (the mass fraction: 20% seed potatoes, 2% glucose, 0.05% glutamic acid, 77.95% distilled water, pH=6.5, sterilization: 121°C, 20 min), in proportion, and the volume was 50 ml/250 ml.

Determination of the molar conversion rate of GABA and cell dry weight

The molar conversion rate of GABA=the content of GABA (mol)/the initial content of L-glutamic acid (mole). The content of L-glutamic acid was measured by a biosensor analyzer (Model SBA-90C), and the content of GABA was measured by the Berthelot colorimetric method. Measurement of dry cell weight first measured the weight of dry centrifuge tube m1, after the fermentation broth was centrifuged, filtered to obtain a wet cell centrifuge tube, 55°C constant temperature drying to constant weight m2, the bacterial cell dry weight m=m1-m2.

Establishment of control experiment group

The seed solution is inoculated into a fermentation medium for fermentation. Fermentation conditions: the room temperature is 25°C, the inoculum is 5%, with natural pH, shaking speed is 150 r/min, and fermentation time is 48 h. Fermentation is carried out three times in a row. GABA conversion rates were 26.56%, 27.01%, 26.02% with an average of 26.53%.

Determination of the best fermentation conditions

Determination of the best inoculation volume: According to the above test results, the 18 h seed solution was inoculated to the fermentation medium in an amount of 2%-10% by volume (every 2% gradient). Cultured at 31°C, rotation speed condition of 170 r/min for 4 d. The relationship between the molar conversion rate of GABA and the inoculum size is as in Table 1.

Table 1. The relationship between inoculum size and conversion rate.

Dinoculum size (%)	2	4	6	8	10
Molar conversion rate (%)	25.01	28.22	31.21	28.83	25.42

As can be seen from the table above, during the inoculation amount of 2% to 6%, with the inoculum size increases, the molar conversion rate of GABA is on the rise. When the inoculum volume is greater than 6%, the molar conversion rate of GABA decreases with the inoculation amount because less inoculation results in a lower overall level of GAD in the broth. Inoculum will make the strain in the early large-scale breeding, resulting in a serious degree of aging in the late fermentation of bacteria to affect the activity of GAD in the fermentation broth [14]. The experiment determined the most suitable inoculum size is 6%.

Determination of the best temperature value: Fermentation temperature on the growth of bacteria, enzyme production and GAD activity play an important role. Based on the previous experiments, this experiment was carried out at 22°C~39°C (a gradient of 3°C) to study the influence of temperature on the molar conversion rate of GABA (Table 2).

Table 2. Relationship between temperature and mycelia dried weight and molar conversion rate.

Temperature (°C)	22	25	28	31	34	37	40
Mycelia dried weight (g/L)	6.23	6.42	6.83	6.90	6.87	5.52	5.20
Molar conversion rate (%)	17.20	23.20	27.21	31.23	27.11	20.02	15.02

Temperature can affect not only the yield and synthesis rate of GAD, but also the activity and composition of GAD by affecting the metabolic activity of bacterial cells [15]. As can be seen from the figure above, when the temperature is between 22°C 31°C, with the increase of temperature mycelia dried weight and GABA molar conversion rate continues to rise; when the temperature is above 31°C, the molar conversion rate of GABA decreased obviously with the increase of temperature, but the mycelia dried weight decreased but was not obvious; both reach their maximum when the temperature is 31°C. Therefore, this experiment to determine the best fermentation temperature of 31°C.

Determination of the best initial pH: The effect of pH on the growth and metabolism of mycelium is mainly reflected in the following aspects: (1) pH can change the charge of nucleic acids, proteins and other substances, thereby changing the biological activity [5]. (2) pH can make the cell membrane charge changes in order to change its ability to absorb nutrients [16]. (3) pH can change the toxic substances in the environment and the availability of nutrients [17]. Different strains have their optimum pH, and strain growth of different periods of its optimum pH value is also different. Due to the difficulty of pH determination and control in the fermentation process of the strain, the initial pH value of 3.7 to 7.3 (a gradient of 0.4) was studied in this experiment. The result is as follows (Table 3).

Table 3. Relationship between pH value and mycelia dried weight and the molar conversion rate of GABA.

pH	3.7	4.1	4.5	4.9	5.3	5.7	6.1	6.5	6.9	7.3
Mycelia dried weight (g/L)	6.25	6.38	6.50	6.71	6.93	6.70	6.49	6.35	6.30	6.24
Molar conversion rate (%)	14.95	18.20	26.10	31.45	26.12	17.20	14.55	8.20	6.52	5.01

It can be seen from the table that when the pH value is less than 4.9, the mycelia dried weight and the molar conversion rate of GABA tend to increase with the increase of the pH value. When the pH value is greater than 4.9, the molar conversion of GABA decreases with the increase of pH. Overall, the molar conversion rate of GABA is the highest at pH 4.5~5.3. It may be because acidic conditions are more conducive to the synthesis of GAD and its enzymatic activity. The optimum pH range of bacterial growth is 4.9~5.7, because in this range the highest mycelia dried weight. Taken together, the optimum initial pH range of fermentation is 4.9~5.3.

Determination of the best shaker speed: Shaker speed is mainly affected by the amount of dissolved oxygen in the fermentation broth to affect cell growth and metabolism [18]. The relationship between the different shaking speed of 90 r/min~210 r/min (a gradient of 20 r/min) and mycelia dried weight and molar conversion rate of GABA is as follows (Table 4).

Table 4. *The relationship between shaking speed, mycelia dried weight and the molar conversion rate of GABA.*

Shaking speed (r/min)	90	110	130	150	170	190	210
Mycelia dried weight (g/L)	4.79	5.02	5.30	5.51	6..21	6.90	6.95
Molar conversion rate (%)	13.20	17.01	22.11	27.22	31.55	27.02	22.42

It can be seen from the table above that when the speed of the shaking table is less than 170 r/min, the mycelia dried weight and the molar conversion of the GABA increase with the increase of the rotation speed of the shaking bed, which is due to the growth metabolism needs a certain amount of dissolved oxygen. However, when the rotating speed was higher than 170 r/min, the molar conversion rate of GABA decreased with the increase of revolving speed because the excessive dissolved oxygen had a certain inhibition effect on GAD production. When the speed is less than 150 r/min, the bacteria can not completely disperse into the fermentation broth, which affects the contact of L-Glu with bacteria and limits the catalytic effect of GAD on L-Glu. Taken together, this experiment to determine the best shaker speed 170 r/min.

Determination of the best fermentation time: Time is also an important factor affecting the microbial fermentation of enzymes. Lack of fermentation time to the accumulation of GAD in the enzyme-producing medium is too small, which is not conducive to the conversion of L-Glu [19]. Fermentation time is too long easy to enter the cell recession senescence or autolysis phenomenon, resulting in the fermentation broth GAD overall activity is not conducive to GABA synthesis [20]. Fermentation time 10~100 h (a gradient of 10 h) and mycelia dried weight and the molar conversion rate of GABA as follows (Table 5).

Table 5. *The relationship between culture time and mycelia dried weight and the molar conversion rate of GABA.*

Fermentation time (h)	10	20	30	40	50	60	70	80	90
Mycelia dried weight (g/L)	1.81	3.75	5.80	6.98	6.94	6.92	6.90	6.55	6.50
Molar conversion rate (%)	2.21	3.25	4.52	4.85	16.20	28.23	32.05	32.03	32.01

It can be seen from the figure above that the mycelia dried weight and the molar conversion rate of the bacteria increased significantly with time within a certain time (10~50 h). 40 h dry cell weight basically no longer increase, indicating that the bacteria gradually enter the stationary phase after 40 h, this time GAD began to accumulate a large number of bacteria. At 50 h, the molar conversion rate increased rapidly and reached the maximum at 60 h, indicating that the amount of GAD reached a maximum at this time. After 60 h mycelia dried weight showed a slight downward trend, when the bacteria gradually into the decay period. Taken together, the optimal fermentation time was 70 h.

Orthogonal test determines the best fermentation conditions

Based on the above single factor experiments, we selected four factors that had a relatively greater effect on the fermentation, and set three levels for each factor to establish the L9 (34) orthogonal test to establish the optimal fermentation conditions for the strain MQ. Experimental design is as follows (Tables 6 and 7).

Table 6. *Orthogonal test factors and levels.*

Factor	(A) Temperature (°C)	(B) Time (h)	(C) pH	(D) Inoculum size (%)
1	30	60	4.9	6
2	31	65	5.1	7
3	32	70	5.3	8

Table 7. *Orthogonal design.*

Test number	Column number				Molar conversion rate (%)
	A	B	C	D	
1	1	1	1	1	29.36
2	1	2	2	2	30.36
3	1	3	3	3	30.31
4	2	1	2	3	30.86
5	2	2	3	1	31.4
6	2	3	1	2	32.03
7	3	1	3	2	29.16
8	3	2	1	3	30.65
9	3	3	2	1	30.2

K1	30.01	29.793	30.68	30.32
K2	31.43	30.803	30.14	30.517
K3	30.003	30.847	30.473	30.607
R value	1.427	1.054	0.54	0.287
The optimal solution	A2	B3	C1	D3

According to the orthogonal test results R (A)>R (B)>R (C)>R (D), the fermentation temperature has the greatest effect on the molar conversion rate of GABA followed by the incubation time, and the effect of inoculum size on conversion is relatively small. The best combination of fermentation conditions by ANOVA was $A_2B_3C_1D_3$. Except for the best value of inoculation amount and the best one obtained from single factor experiment analysis, the best value of other factors is consistent with the result of single factor test. Taking into account the interaction between the various factors and the inoculum size has little effect on the conversion rate, the results of the orthogonal experiment are more accurate. The optimum fermentation conditions were as follows: fermentation temperature 31°C, fermentation time 70 h, pH value 4.9, inoculation 18 h, inoculation 8%, shaking speed 170 r/min. According to these conditions, the transformation experiment was conducted. The GABA conversion rate of strain MQ reached 32.03%. Under this condition, the average fermentation time was 32.06%.

Result Analysis

After optimization of fermentation conditions, the molar conversion of GABA was 32.06%, which was 20.84% higher than that of 26.53% before optimization. This is the highest yield of GABA obtained by *Absidia*. The preparation of GABA under this condition can greatly improve the utilization rate of raw materials and provide a theoretical basis for further industrial production.

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