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Optimization of C/N ratio preparation of protein-rich and multi-enzymes feed thallus through synergic fermentation of mixed distillers' grains

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Abstract: A new procedure of determining optimal C/N (the rate of carbon source to nitrogen source) of mixed distillers' grains for combined bacteria synergic fermentation is established. At the same time an improved method evaluating bacteria growth, called method of dry cell weighing by filtering is developed. For each combination of C and N, their initial and residual contents before and after fermentation respectively are determined. Then followed the calculation of utilization of C and N sources by the compound bacteria. The optimal C/N is finally located from among the utilization of C and N of several combinations and the weight of produced mass of oven-dried thallus. The conditions of fermentation are: inoculum size 10%, temperature 30.0℃, rotational speed 170 r/min, shake culture time 48h. The best results obtained from orthogonal experiments are: maximum mass of oven dried thallus is 14.693g in a liter liquid medium, maximum utilization rate of carbon source is 98.13% and maximum utilization rate of nitrogen is 78.14%. Optimal C/N is 5.1.

Keywords: optimal C/N; fermentation distillers' grains; utilization rate of carbon source; transformation rate of nitrogen source

Introduction

The conversion of mixed distillers' grains into thallus feed with protein-rich and multi-enzymes by fermentation is an important approach to utilize brewery waste streams. One of the crucial techniques is to carry out fermentation at an optimal C/N ratio with high-quality strain, so that a high-quality thallus feed with protein-rich and multi-enzymes could be produced.

All the previous references had few reports on determining method in regard to optimal C/N ratio. There was a little applied problem on C/N ratio in some related papers. The adoptive method was only that urea or $(NH_4)_2SO_4$ was added. And according to the volume that urea or $(NH_4)_2SO_4$ was added and the volume that carbon source was determined, C/N radio was estimated (Liang, 1997). Our study on determining method with regard to optimal C/N ratio was made good use of orthogonal experiments. And through changing the original additive volume of carbon source or nitrogen source, we obtained the most mass of thallus growth, the most rate of utilization of carbon source and the most rate of transformation of nitrogen source. According to the three indexes, optimal C/N ratio can be determined. The characteristics of determination are that both method and operation are simple, the determining time is short and accuracy is high.

1 Materials and procedures

1.1 Materials

Strains #8503 and #8505 are taken from the stock of our research group (Hou, 1999). Media are Malt Extract Agar (Chen, 1996) and Potato Glucose Agar ATCC, 1997 (Chen, 1996). For the preparation of mixed distillers' grain fluid medium, distillers' grain of white spirit (about 60% water content) was added into distillers' grains of alcohol (about 95% water content), such that the content of solid substance in mixed distillers' grains reached 13%. An all non-thallus solid substances were removed after thorough mixing by filtration. The filtrate was reserved as a fluid medium after sterilization.

1.2 Analytical methods

The concentration and utilization rate of reducing sugar were determined spectrophotometric ally by the 3,5-dinitrosalicylic acid method (Zhang, 1986).

The concentration and transformation rate of nitrogen were determined by the spectrophotometric phenate method (Lu, 1996).

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Determination of protein follows the National Standards of People's Republic of China, Analysis method of protein in foodstuff GB5009 – 85 (National Standards of P. R. China, 1985).

Determination of the amount of mass thallus growth: The difference between the mass of oven dried thallus after fermentation and that of initial oven dried thallus is the mass of oven dried thallus growth. In other words, the mass of oven dried thallus growth is the difference between the mass of dry cell weighing after fermentation and that of dry cell weighing of 10 ml inoculum.

2 Determination of optimal C/N

2.1 Design of orthogonal experiment (Probability and Statistics Teaching and Research Group, 1979; Gao, 1988)

Lg(3^4) orthogonal table, in which orthogonal factor and levels are designed. Experiments of orthogonal interactions between the carbon and nitrogen source, and their three levels are then studied (Table 1).

Table 1 Design of orthogonal factor and level (Ⅰ and Ⅱ)

	Ⅰ			Ⅱ			
Factor	C Carbon source, g/L	N Nitrogen source, g/L	Factor	C Carbon source, g/L	N Nitrogen source, g/L	E C × N	F C × N
Level Ⅰ	6	2	Level Ⅰ	15	2		
Level Ⅱ	10	3	Level Ⅱ	25	3		
Level Ⅲ	14	4	Level Ⅲ	35	4		

2.2 Operation of orthogonal experiment and determination of related indices

Put 150 ml of soaking fluid of mixed distillers' grains into a 500 ml cone bottle. With the contents of sources set in the above Lg(3^4) orthogonal tables, each experiment is carried out twice with like samples. All media are sterilized under the pressure 1.03×10^5 Pa and the temperature 121℃ for 15 min in an autoclave. When the pressure is reduced to zero, remove the medium from autoclave and cool it quickly to 30℃. Then take out 50 ml of the sterilized culture fluid for determination of its concentrations of carbon source (indicated by reducing sugar) and nitrogen source (indicated by $(NH_4)_2SO_4$) which are considered as the initial values before fermentation. Meanwhile, inoculate the remained 100 ml of culture fluid with strain 8503 and 8505 of 10% size that is 10 ml for each strain, and shake culture at temperature 30.0℃, rotational speed 120 r/min, for 48h. After fermentation take out the bottle and filter the mixture quickly. First determine mass of thallus by method of weighing dry cell by filtering. Then determine the concentration of carbon source (reducing sugar) and nitrogen source ($(NH_4)_2SO_4$) of filtrate, and calculate their utilization rate. The determination indices, related to concentration of carbon and nitrogen sources and their utilization rate, can be proceeded by the following formulas:

The reducing sugar (mg) is calculated by regression equation of standard curve which is determined spectrophotometric ally by the 3,5-dinitrosalicylic acid method, thus,

$$\text{reducing sugar(mg)} = 0.8955\ OD_{520} + 0.0618,\ \text{dependent coefficient: } r = 0.9991. \quad (1)$$

The calculation formula of concentration of carbon source is:

$$C(\text{g/L}) = (\text{milligram number of reducing sugar/milliliter}) \times \text{dilute multiple}. \quad (2)$$

The calculation formula of utilization rate of carbon source is:

$$\eta_c(\%) = [(\text{initial concentration of reducing sugar-final concentration of reducing sugar})/\text{initial concentration of reducing sugar}] \times 100\%. \quad (3)$$

The $(NH_4)_2SO_4(\mu g)$ is calculated by regression equation of standard curve which is determined the spectrophotometric phenate method. Thus,

$$(NH_4)_2SO_4(\mu g) = 199.4079\ OD_{625} - 1.6333,\ \text{dependent coefficient: } r = 0.9998. \quad (4)$$

The calculation formula of concentration of nitrogen source (indicated by $(NH_4)_2SO_4$) is:

$$(NH_4)_2SO_4(\text{g/L}) = ((NH_4)_2SO_4\ \mu g \text{ number/milliliter}) \times \text{dilution multiple} \times 10^{-3}. \quad (5)$$

The calculation formula of transformation rate of nitrogen source is:

η_N(%) = [(initial concentration of $(NH_4)_2SO_4$ − final concentration of $(NH_4)_2SO_4$)/initial concentration of $(NH_4)_2SO_4$] × 100%. (6)

2.3 Experiments of interaction between carbon and nitrogen sources

In order to make a further study on (1) the effects on the mass of thallus growth; (2) the effects of thallus on the utilization rate of carbon and transformation rate of nitrogen source; (3) to determine whether or not there is any the interaction between carbon source and nitrogen source and (4) on the effects of interactions on the extent of fermentation of the culture fluid of mixed distillers' grains, some interaction experiments between C and N sources are carried out on the bases of $Lg(3^4)$ orthogonal factors and levels, with mass of thallus growth and utilization rate of carbon sources and transformation rate of nitrogen sources used as examining indices. The results obtained are then subjected to range analysis (Table 2).

Table 2 Results of $Lg(3^4)$ orthogonal experiment II and orange analysis

Column No. Experiment No.	1 Carbon concentration(C)	2 Nitrogen concentration(N)	3 E(C × N)	4 F(C × N)	G Mass of oven dry thallus	η_C Utilization rate, %	η_N Transformation rate, %
1	Ⅰ	Ⅰ	Ⅲ	Ⅱ	1.4693	96.10	78.14
2	Ⅱ	Ⅰ	Ⅰ	Ⅰ	1.4601	97.04	76.66
3	Ⅲ	Ⅰ	Ⅱ	Ⅲ	0.7399	97.49	70.54
4	Ⅰ	Ⅱ	Ⅱ	Ⅰ	0.7461	94.83	68.99
5	Ⅱ	Ⅱ	Ⅲ	Ⅲ	0.7530	97.99	64.14
6	Ⅲ	Ⅱ	Ⅰ	Ⅱ	0.7462	98.31	64.82
7	Ⅰ	Ⅲ	Ⅰ	Ⅲ	0.7536	94.89	66.99
8	Ⅱ	Ⅲ	Ⅱ	Ⅱ	0.7493	97.02	63.23
9	Ⅲ	Ⅲ	Ⅲ	Ⅰ	0.7488	97.71	68.89
$I_{j,G}/3$	0.9897	1.2231	0.9866	0.9850			
$Ⅱ_{j,G}/3$	0.9875	0.7484	0.7451	0.9883			
$Ⅲ_{j,G}/3$	0.7450	0.7506	0.9904	0.7488			
$R_{j,G}$	0.2447	0.4725	0.2415	0.2395			
$I_{j,C}/3$	95.27	96.88	96.75	96.65			
$Ⅱ_{j,C}/3$	97.35	97.04	96.45	96.43			
$Ⅲ_{j,C}/3$	97.83	96.54	97.27	96.73			
$R_{j,C}$	2.56	0.50	0.83	0.36			
$I_{j,N}/3$	71.37	75.10	69.49	71.51			
$Ⅱ_{j,N}/3$	68.01	65.98	67.59	68.73			
$Ⅲ_{j,N}/3$	68.08	66.37	70.39	67.22			
$R_{j,N}$	3.36	9.12	2.80	4.29			

According to the results of $Lg(3^4)$ orthogonal experiments I and the range analyses of G (mass of oven dried thallus growth), we can see clearly that carbon source is the main factor affecting the mass of thallus growth. Among the various combinations of C and N, the tendency of variation of G is that for a given N, G is always from low to high. So concentration of carbon source must be increased. There for orthogonal experiment II is designed, where the three levels of carbon source are 15 g/L, 25 g/L and 35 g/L, respectively (Table 1).

According to the results of orthogonal experiment II in Table 2 and by the range analysis of G it is clear that nitrogen source is the main factor affecting G, while carbon source is the minor one. The highest mass of G is produced by C_1N_1 combination, which is as content G is concerned.

The range analysis of η_c shows that the carbon source content is the main factor affecting η_c. Although the highest utilization rate of carbon source is resulted from C_3N_2 combination being slightly higher than C_1N_1 by 2.21%, its mass of oven dry thallus G value is considerably lower than that of C_1N_1 by the optimal combination about 50%. As a combined result, C_1N_1 is obviously the best choice.

The range analysis of η_N indicates that there is not much difference between ranges of carbon source and that of nitrogen source. So both of them are important. And there is obvious interaction between these two factors. The highest transformation rate of nitrogen source is also obtained from C_1N_1 combination.

3 Experimental result of optimal C/N

According to the statistical analysis of the three indices of orthogonal experiment Ⅰ and Ⅱ, the conclusion drawn is that the carbon source addition in orthogonal experiment Ⅰ is rather low, while that of $(NH_4)_2SO_4$ is acceptable. Therefore the carbon source addition is increased in orthogonal experiment Ⅱ. Based on analyses of direct experiment results and of range and interaction of these two experiments, along with the cost reduction and raw – materials saving, it is thought that C_1N_1 is the optimal combination which means that there are 15g of carbon source and 2g of $(NH_4)_2SO_4$ to be added into each liter of cultural fluid. Apparently the C/N rate could now be calculated from the addition shown by C_1N_1 and would be equal to 7.5. However, since the initial contents of carbon source (indicated by reducing sugar) and nitrogen source, which is respectively 0.8150 g/L and 1.115 g/L, should be considered together with the additions of carbon source and $(NH_4)_2SO_4$, in order to obtain an actual C/N, the optimal C/N thus calculated is equal to 5.1 instead of 7.5. This is the practical optimal C/N for fermentation about distillers' grains.

In order to make a further saving of raw material so as to reduce cost, it is desirable to first decompose the amylum in distillers' grains by use of amylase into sugar witch then can be utilized by microbe. At the same time auxiliary raw material wheat bran is added to replace carbon source addition. As a result the cost will be further reduced and the benefit gained will be maximum.

4 Discussion and conclusion

By determining the initial and residual N contents in culture liquid respectively before and after fermentation, and by adjusting the initial C/N rate through orthogonal experiments I and II, an optimal C/N is established meanwhile an improved method of weighing dry call is developed.

In this paper, according to the orthogonal experiment Ⅰ and Ⅱ, C_1N_1 is found to be the optimal combination for getting maximum amount of oven dry thallus. Under combination of C_1N_1 in each of 100 ml of cultural fluid, the amount of mass of oven dry thallus reached is the highest 1.4693g. The transformation rate of nitrogen source is also the highest 78.14%. The utilization rate of carbon source is 96.1%, which is less than C_3N_2 only by 2.21%. So through synthetic analysis, it is concluded that C_1N_1 is the optimal combination, of which the optimal C/N is 5.1.

In this experiment, by deducting residual nitrogen content after fermentation from the initial content in cultural fluid, the net proportion of nitrogen transformed is determined which should have been absorbed, transformed and synthesized by bacteria. In orthogonal experiment Ⅰ, the content of thallus protein of experimental No.2, 3, 6 and 9 are determined, they are 56.30%, 55.94%, 53.07% and 52.97% respectively and their average value is 54.57%. So the transformation rate of the inorganic nitrogen by the strains is quite obvious. In this way, the biomass of thallus growth can quickly increase. The final outcome is that the bio-protein content will be increased greatly in the distillers' grains.

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