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Research Article

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Optimization of conditions for glucoamylase, α-amylase and acidic protease production by *Aspergillus Oryzae* Koji

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ABSTRACT

In this experiment, indica rice was used as raw material to koji-making, the koji-making conditions of Aspergillus oryzae were optimized and the effects of inoculation temperature, inoculation quantity, culture humidity, culture temperature, and culture time on enzyme activity were studied. The results indicated that the optimum koji-making conditions were as follows: inoculation temperature at 35 ℃, 0.25% inoculation quantity, culture humidity at 75%, culture temperature at 37 ℃, 56 h culture time. Under the above conditions, the glucoamylase activity and α-amylase (liquefying enzyme) activity could reach up to 475.52 u/g koji and 177.12 u/g koji respectively. When the culture temperature was 33 ℃, acidic protease activity reached a maximum of 59.68 u /g.

Key words: *Aspergillus oryzae*; koji-making; glucoamylase; α-amylase; acid protease; activity

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INTRODUCTION

Aspergillus oryzae is a kind of aerobic type fungi. It belongs in *Deuteromycotina*，*Hyphomycetes*, *Moniliales*, *Moniliaceae* and *Aspergillus*, and it is one of 40 safe microbial strains that American FDA had published [1], and has extremely important application in food brewing industry. *Aspergillus oryzae* is characterized by producing many kinds of enzymes [2-3], and high enzyme activity, fast growth, strong adaptability, not producing toxins, and being easy to manage. It is an important microorganism in the wine industry and widely distributed in a variety of koji [4]. In the brewing process, starch is converted into glucose by *Aspergillus oryzae*. Therefore, the decomposition degree of raw materials was determined by the enzyme activity to a large extent [5]. The koji of sake is made by using *Japonica* rice, Strains are *Aspergillus oryzae*, koji-making time is about 48h, rice are used in an amount of 20% of the total amount of raw materials. In Chinese Huangjiu brewing, wheat koji or indica is mostly used as the material to koji-making, strains are *Rhizopus*, starter-making time is long [6]. In this experiment, indica rice is used as raw material, Aspergillus oryzae is the only strain. Through exploring inoculation temperature, inoculation quantity, culture humidity, culture temperature, and culture time, studying on the effect of koji-making conditions of *Aspergillus Oryzae* on enzyme activity, on this account, expecting to reduce the amount of koji usage in brewing production, helping improve economic efficiency, and at the same time offering help to solve pure fermentation, mechanized production.

EXPERIMENTAL SECTION

Strains: *Aspergillus oryzae* was kept in liquor making laboratory Room 528, College of Bioengineering, Sichuan University of Science and Engineering and potato dextrose agar (PDA) medium (with 20% potato, 2% glucose and 2% agar) was used for culture. Indica rice was purchased from local market.

Koji-making process: Indica rice was purchased from the local market. Raw rice samples were washed and soaked for more than 12 h and then drained in water for some time. The samples were then steamed in the autoclave (110℃) for 40min and the temperature was reduced to suitable values. Each fungal spore was inoculated separately and

cultured at appropriate conditions [7], koji turnover at intervals of 12h.

Glucoamylase activity assay [8，9]: Weighing 2g dry koji, then crushed by a Muller. The crushed sample (2g) was extracted with (36-2×moisture%) ml water and 4ml buffer for 1h at 40℃ water bath, stirring once every 15min, then filtering with a dry filter paper, discarding the first 5ml, receiving remain clear to use. Glucoamylase activity was measured by using the method of GB 8276-2006 food additive provisions glucoamylase preparations.

Liquefying enzymatic activity assay [8，10]**:** Weighing 2g dry koji, then crushed by a Muller. The crushed sample (2g) was extracted with (10-2 × moisture%) ml buffer for 1h at 40℃ water bath, stirring once every 15min, then filtering with a dry filter paper, discarding the first 2ml, receiving remain clear to use. Liquefying enzyme activity was assayed by using the method of GB 8275-2009 food additive provisions α-amylase preparations.

Acidic protease activity assay [8，11]**:** Weighing 2g dry koji, then crushed by a Muller. The crushed sample (2g) was extracted with(20-2×moisture%) ml buffer for 30min at 40℃ water bath, stirring once every 15min, then filtering with a dry filter paper, discarding the first 2ml, receiving remain clear to use. Acidic protease activity was measured by using the method of GB 23527-2009 food additive provisions protease preparations.

Single factor experiment: 30g of indica rice(which was soaked and drained) were used as the material in 250ml flask，then the samples were then steamed in the autoclave (110℃) for 40min and the temperature was reduced to suitable values. Each fungal spore was inoculated separately and cultured at appropriate conditions [7].This experiment included a total of five factors: inoculation temperature, culture humidity, inoculation quantity, culture temperature, culture time, respectively. The inoculation temperature was 25 ℃, 30 ℃, 35 ℃, 40 ℃, 45 ℃, culture humidity was 60%, 70%, 80%, 90%, inoculation quantity was 0.1%, 0.25%, 0.5%, 0.75%, 1.0%(w/w), culture temperature was 27 ℃, 31 ℃, 35℃, 39 ℃, 43 ℃, culture time was 38h, 43h, 48h, 53h, 58h, respectively, *Aspergillus oryzae* seed koji was inoculated separately and cultured at 31℃, 85% relative humidity for 24h, then changing the culture conditions, training to the end, koji turnover at intervals of 12h. Enzyme activity was measured after culturing.

Orthogonal experimental design [12]: On the basis of single factor experiments, in order to optimize enzyme activity of koji, considering culture humidity, temperature, time to be the main factors. Specific levels of the factors L9 (3^4) was shown in Table 1.

Table1 Factors and levels of the test

level	humidity /%	temperature /℃	time /h
1	65	33	50
2	70	35	53
3	75	37	56

RESULTS AND DISCUSSION

Effect of inoculation temperature on the enzymatic activity of koji

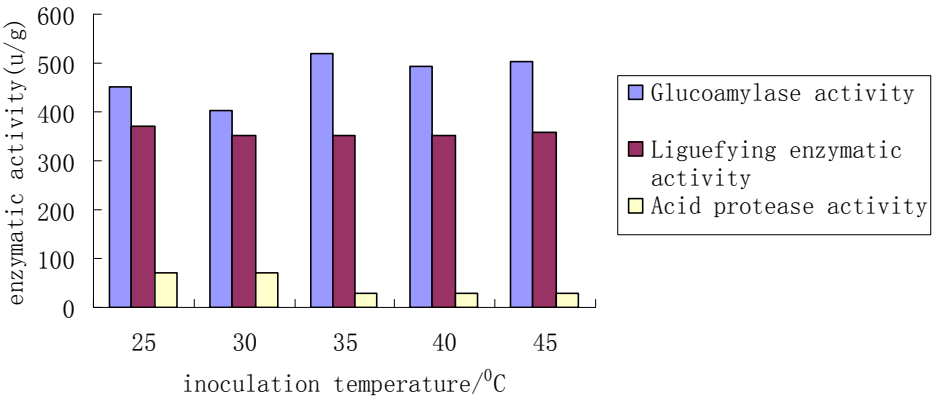

Fig.1. Effect of inoculation temperature on the enzymatic activity of koji

Inoculation temperature for *Aspergillus oryzae* should below 40℃, the ideal inoculation temperature is 32±2 ℃[13].

As we can seen from Fig.1, when the temperature was 35℃, glucoamylase activity was the highest, liquefying enzymatic activity did not change significantly with inoculation temperature, protease activity decreased when the temperature raised.

Effect of culture humidity on the enzymatic activity of koji

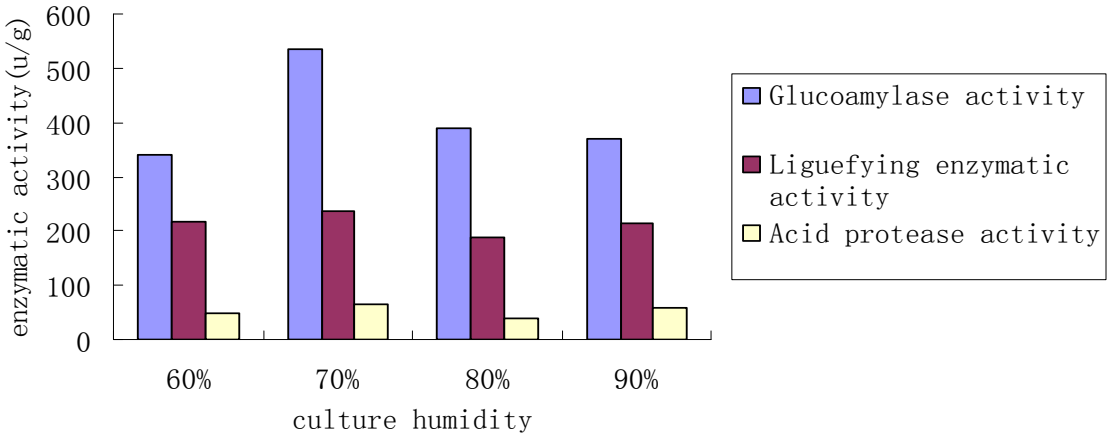

Fig.2 Effect of culture humidity on the enzymatic activity of koji

Each of the microorganisms has certain requirements for water, when it deviates from the optimum requirements, microbial growth is inhibited. Water activity for *Aspergillus oryzae* is 0.80 to 0.88[13], as can be seen from Fig.2, with the relative humidity increasing, three enzymes activity increased firstly, but then reduced. The result showed that low culture humidity might affect *Aspergillus oryzae* growth, and three enzymes activity reached a maximum at 70% culture humidity.

Effect of inoculation quantity on the enzymatic activity of koji

When inoculation quantity is small, there will be lack of biomass, and nutrient medium is excess, when inoculation quantity is overdose, the nutrient medium is limited, it is difficult to maintain the growth of *Aspergillus oryzae*, forming a large number of dormant spores, reducing enzyme activity [5]. From Fig.3, the activity of three enzymes did not change significantly with inoculation quantity. It might be because a small amount of inoculums had little effect on the results.

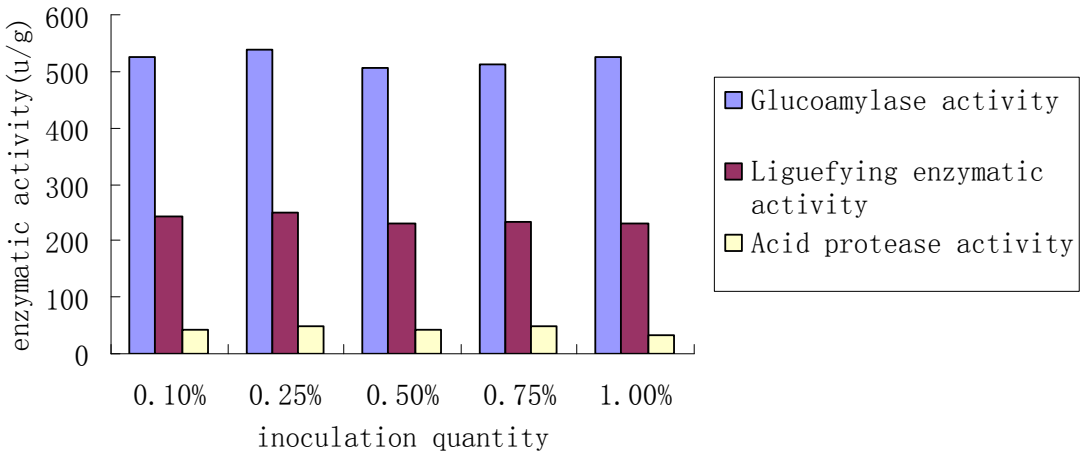

Fig 3 Effect of inoculation quantity on enzymatic activity of koji

Effect of culture temperature on the enzymatic activity of koji

The ideal temperature for *Aspergillus oryzae* growth is 30℃ ~ 35℃, bellowing 25℃, growth slow, above 40 ℃, growth difficulty [13], only the culture temperature within the suitable range, it can promote microorganisms growth and improve enzyme activity. Fig. 4 shows that the impact of temperature on enzyme activity was significant, when temperature was 35℃, glucoamylase activity was highest, then reduced with temperature, while protease activity was higher at lower temperatures.

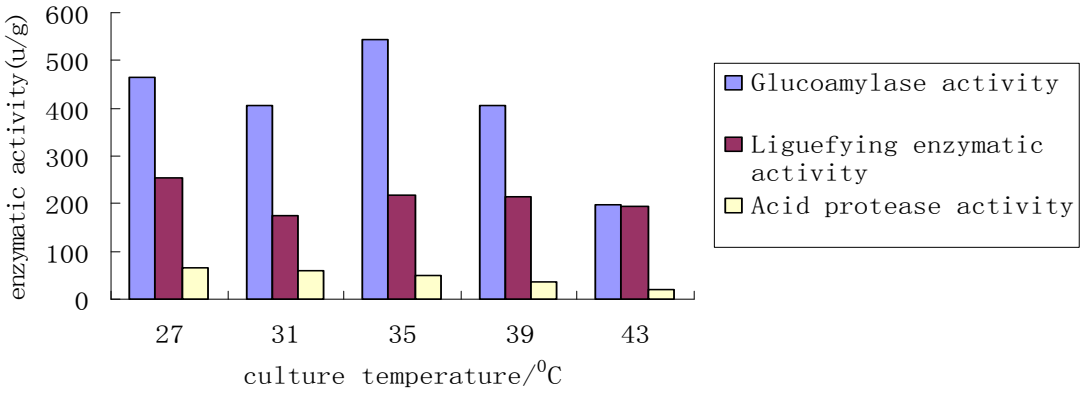

Fig .4 Effect of culture temperature on the enzymatic activity of koji

Effect of culture time on the enzymatic activity of koji

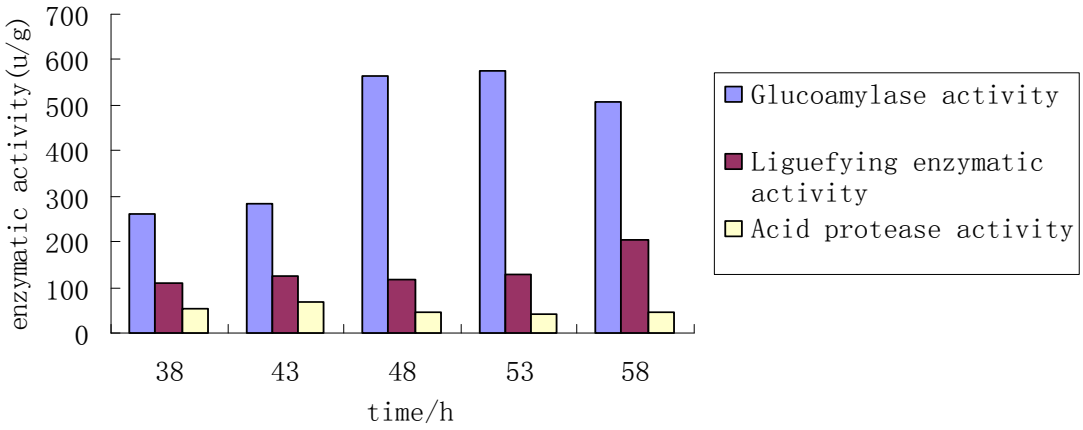

Fig.5 Effect of culture time on the enzymatic activity of koji

As we can seen from Fig.5, in the initial culture period, there was a certain adaptation period for *Aspergillus oryzae*, so they grew slowly, and the activity was low too. As the extension of time, activity increased and reached a peak, glucoamylase activity was earlier than the liquefying enzymatic activity to reach to the maximum.

The results of orthogonal experiments

Table 2 Method and results of orthogonal experimental design

Test No	A（humidity）	B (temperature）	C (time)	D (empty）	Glucoamylase activity	Liquefying enzymatic activity	Acidic protease activity
1	1	1	1	1	246.483	111.63	45.135
2	1	2	2	2	173.988	124.08	29.344
3	1	3	3	3	281.281	139.77	39.416
4	2	1	2	3	147.89	115.53	34.604
5	2	2	3	1	289.98	134.01	35.561
6	2	3	1	2	231.84	133.41	39.291
7	3	1	3	2	434.7	134.91	58.315
8	3	2	1	3	318.978	117.18	40.037
9	3	3	2	1	469.476	140.82	55.766

Table 3 The results of intuitive analysis by using the date in table 2

Name	R-values			
R(glucoamylase activity）	184.48	66.55	71.54	85.93
R(liquefying enzymatic activity）	5.81	17.31	15.49	6.64
R(acidic protease activity）	14.89	11.04	4.53	7.47

As we can be derived from Table 3, the optimization of koji-making conditions with indica rice of *Aspergillus*

oryzae was as follows: temperature 37 ℃, relative humidity 75%, time 56 h. The koji quality was good with high enzyme activity, where the glucoamylase activity was 470.52 u/g, the liquefying enzymatic activity was 177.12 u/ g, respectively. When the culture temperature was 33℃, acidic protease activity reached a maximum of 59.68 u/g of koji.

CONCLUSION

(1) Orthogonal test results shows that, the order of affecting factors of glucoamylase activity was: relative humidity > time > incubation temperature; the order of affecting factors of liquefying enzymatic activity was: incubation temperature > time > relative humidity; the order of affecting factors of acidic protease activity was: relative humidity > incubation temperature > time.

(2) The optimization results of koji-making conditions were as follows: inoculation temperature at 35℃, 0.25% inoculation quantity, culture humidity at 75%, culture temperature at 37℃, 56 h culture time. Under the above conditions, the glucoamylase activity and liquefying enzyme activity could reach up to 475.52 u/g and 177.12 u/g respectively. When the culture temperature was 33 ℃, the acidic protease activity of koji reached a maximum of 59.68 u/g.

(3)By optimizing the conditions of koji-making, we can reduce the amount of koji usage in the production, thus help to improve the economic efficiency.

Acknowledgments

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